Synaptic dysfunction in the perirhinal cortex of the R6/1 mouse model of Huntington's disease

Thesis

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Synaptic dysfunction in the perirhinal cortex of the R6/1 mouse model of Huntington's disease

Damian Michael Cummings, B.Sc. Honours.

Department of Biological Sciences

Faculty of Science

The Open University

This dissertation is submitted for the degree of

Doctor of Philosophy

Submitted 21st September 2004

Submission date: 21 September 2004
Award date: 1 December 2004

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Declaration

This dissertation is the result of my own work.

This thesis does not exceed 100, 000 words, including bibliography.

Parts of this work have been published in abstract form (Cummings *et al.*, 2002; 2003a; 2003b; 2003c; 2003d; 2004; Milner *et al.*, 2004a) and are currently in preparation for full journal publication.

Abstract

Huntington's disease (HD) is a fatal brain disorder characterised by a progressive motor, psychiatric and cognitive decline, often manifesting in midlife. Since the identification of the gene responsible for HD and the development of predictive testing, it has become apparent that asymptomatic patients often exhibit early cognitive deficits. In particular, an impairment in recognition memory that is evident prior to the onset of classical symptoms and frank cell death. The perirhinal cortex is believed to be involved in processing aspects of recognition memory, specifically the discrimination between novel and familiar cues. Evidence suggests that activity-dependent decrements in neuronal activity within the perirhinal region could underlie this cognitive process and that the synaptic mechanism may be one of long-term depression (LTD).

In the R6/1 mouse model of HD, synaptic plasticity is progressively altered in the perirhinal cortex *in vitro*. LTD expression is enhanced at one month, but then declines and is absent by 7 months of age. Dopamine is shown here to be a neuromodulator at normal perirhinal cortical synapses. Fluorescent immunohistochemical techniques are used to demonstrate altered dopamine receptor expression in the R6/1 perirhinal cortex that correlates with the aberrant synaptic plasticity. By exposing normal mouse slices to a D_2 dopamine receptor antagonist, it is possible to recapitulate the R6/1 perirhinal synaptic phenotype. Importantly, the alteration of synaptic function and loss of LTD in R6/1 mice can be restored by applying an agonist of dopamine receptors to brain slices.

These data suggest that impaired perirhinal LTD may contribute to poor recognition memory and that the defect is at the level of the dopaminergic system. Moreover, dopaminergic therapy, targeted to the cortex, may be beneficial in HD.

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List of Abbreviations

3-NP	3-nitropropionic acid
5-HT	5-hydroxytryptamine (serotonin)
AC	Adenyl cyclase
ACSF	Artificial cerebrospinal fluid
AIDA	UPF 523/(RS)-1-aminoindan-1,5-dicarboxylic acid
AMPA	a-amino-3-hydroxy-5-methyl-4-isoazolepropionate
AP5 (or APV)	(D,L)-2-amino-5-phosphonopentanoic acid / (D,L)-2-amino-5-phosphonovalerate
ATP	Adenosine triphosphate
BCM	Bienenstock, Cooper & Munro (theory)
BMI	(-)-bicuculline methiodide
CAG	Cytosine-Adenine-Glutamine
CaMKII	Calcium/calmodulin dependent protein kinase type II
cAMP	Cyclic 3',5'-adenosine monophosphate
CBP	CRE binding protein
cGMP	Cyclic guanine monophosphate
CMV	Cytomegalovirus
CNQX	6-cyano-7-nitroquinoxaline-2, 3-dione disodium
CNS	Central nervous system
CPPG	(RS)-a-cyclopropyl-4-phosphonophenylglycine
CRE	Cyclic AMP response element
CREB	cAMP response element binding protein
CS	Conditioned stimulus
DCG-IV	(2S, 2'R, 3'R)-2-(2',3'dicarboxycycloproyl)glycine
DHPG	(R,S)-3,5-dihydroxyphenylglycine
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EPSP	Excitatory postsynaptic potentiation
GABA	γ-aminobutyric acid
HAP	Huntingtin associated protein
HD	Huntington's disease
Hdh	Mouse HD gene homologue
HIP	Huntingtin interacting protein
HRP	Horseradish peroxidase
KA	Kainic acid/kainate
L-AP4	(S)-2-amino-4-phosphono-4-phosphonobutanoate
LFS	Low-frequency stimulation
LTD	Long-term depression
LTP	Long-term potentiation
LY 341495	(2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl)-propanoic acid
MAPK	Mitogen activated protein kinase
MCPG	(RS)-a-methyl-4-carboxyphenylglycine
Mepp	Miniature endplate potential
mGlu	Metabotropic glutamate

MSSN	Medium-sized spiny neurone
NaOH	Sodium hydroxide
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthetase
PB(S)	Phosphate buffered (saline)
PD98059	2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one
PET	Positron emission tomography
PKA	cAMP-dependent protein kinase A
PKC	Calcium/phospholipid dependent protein kinase C
PKG	NO-cGMP dependent protein kinase G
PLC	Phospholipase C
PMT	Photomultiplier tube
PPD	Paired-pulse depression
PPF	Paired-pulse facilitation
PTX	Picrotoxin
Q	Glutamine
QP	Quinpirole hydrochloride
SCH23390	(R)-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride
SCH50911	(2S)-(+)-5,5-dimethyl-2-morpholineacetic acid
SE	Superficial entorhinal
SKF38393	(±)-1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol hydrobromide
Sp1	Specificity protein 1
SPECT	Single photon emission computerised tomography
ST	Superficial temporal
TBS	Theta burst stimulation
TSA	Tyramide signal amplification
US	Unconditional stimulus
VSCC	Voltage sensitive calcium channel
YAC	Yeast artificial chromosome

1. Introduction

1.1 Overview

Huntington's disease (HD) is a genetic and fatal neurodegenerative disorder, commonly characterised by a midlife decline in motor and cognitive skills. Following the identification of the gene and the development of predictive testing it has been realised that, many years prior to the onset of the classical symptoms, subtle cognitive deficits are apparent. In particular, impairments in the ability to discriminate between novel and familiar cues (recognition memory) are readily discernable. The first part of this introduction will describe the symptoms of HD and the mechanisms identified to date that are considered to be involved in this devastating disorder. It also summarises the available animal models used to study HD. The second part discusses synaptic plasticity; the cellular mechanism widely believed to underlie learning and cognitive processing. Studies describing changes in hippocampal synaptic plasticity in mouse models of HD are described in the third part. Given the impairment in recognition memory detectable in asymptomatic HD patients, the final part of the introduction describes the perirhinal cortex, a cortical brain region thought to be involved in the process of novelty discrimination.

1.2 Huntington's disease

Huntington's disease is a fatal neurological disorder affecting approximately three in every 10, 000 of the population. The disease is named after Dr. George Huntington, M. D. (figure 1.2.1), who, in 1872, presented a comprehensive report on this disorder (Huntington, 1872). Although our understanding of the disease has increased dramatically since its identification, a cure has yet to be found and current approaches to treatment are similar to that used in the 19th century; they remain palliative and not curative. The disease is a genetic condition and is inherited in an autosomal dominant fashion. Symptoms usually manifest in the third to fourth decade of life, often after the individual has unknowingly passed on the defective gene to their offspring. Classical symptoms include a loss of motor control and psychological changes. The loss of motor control is characterised by dance-like movements, known as chorea (derived from the Greek *χορός* (choros), to dance), after which the disease was originally called. (Huntington's chorea has recently been renamed Huntington's disease when it was recognised by clinicians that almost half of cases present with other symptoms and the absence of choreatic movements; the term Huntington's disease therefore describes the full syndrome rather than just one symptom.) Upon *post-mortem* examination massive cell death is observed, particularly within the striatum and cerebrum, resulting in enlargement of the ventricles and thinning of the cortical mantle respectively. The identification of the gene has enabled predictive testing and it is now apparent that, prior to the onset of overt symptoms and cell death, there is a subtle decline in cognitive functions and it is possible that alterations in the communication between neurones may underlie these changes in cognition.

1.2.1 An historical background

Reports of hereditary chorea date back as early as 1832 (see Harper, 2002), however the first fully descriptive report of the disorder that was later to become known as Huntington's chorea was in a letter by Charles Oscar Waters (1841). In his letter Walters describes a chorea with a hereditary nature and how it progresses, ultimately leaving the patient demented. Another detailed description, this time from Europe, was published in 1860 (Lund). This report was originally overlooked as it was written in Norwegian and its importance has only come to the fore since its translation in 1959 (Ørbeck). Despite these and other reports, it was George Huntington's paper, published in the Philadelphia journal *The Medical and Surgical Reporter* in 1872 (Huntington, 1872), that caught the attention of other medical practitioners. The first part of the report contains a general description of non-hereditary forms of chorea, their causes and treatments available at the time. The second part describes a hereditary form of chorea. This account was in fact a concise and comprehensive portrayal of the condition later to bear Huntington's name. The manuscript describes the adult onset, progressive nature and fatal outcome; the choreic movements and psychological impairments; the effect socially on the patient and family; and the pattern of inheritance. Huntington had drawn not only on his own observations, but that of his father and grandfather, both of whom had held practices in Long Island spanning over 60 years. It is perhaps the presence of verifiable cases in Huntington's report, along with the brief yet accurate description of the disease, which made this work the seminal paper that it has become.

"... an heirloom... hardly ever manifesting until middle life, and then coming on gradually but surely until the hapless sufferer is but a quivering wreck of his former self."

Figure 1.2.1: Dr. George Huntington (1850-1916) (*top*). (Reproduced from Bates *et al.*, 2002). *Bottom:* A quote from George Huntington's report, published in 1872, which describes the devastating nature of the disorder later to bear his name.

Over the next century our knowledge of HD did progress, however the isolation of the gene in 1993 (Huntington's Disease Collaborative Research Group) sparked an exponential increase in our understanding of the processes involved in the early stages of the disease. Huntington's disease is caused by a mutation in the gene that encodes the protein huntingtin, the function of which is still unclear, although there are indications that it is involved in regulation of gene transcription, vesicle transport and regulation of processes occurring at the synapse. Near to the amino terminal of the gene in exon 1 is found a cytosine-adenine-guanine (CAG) repeat that encodes a tract of glutamines (Huntington's Disease Collaborative Research Group, 1993). When the number of glutamines exceeds a threshold the disease will develop at some point in the individual's lifetime. Interestingly the length of this expanded repeat determines, to a degree, the age of onset and the severity of the disease; longer repeats give rise to an earlier, more aggressive syndrome with very high repeat numbers resulting in a pernicious juvenile form.

Prior to the identification of the gene, the best animal models of the disease were based on recapitulating the pattern of cell loss using chemical lesions in rats (for review see Yohrling IV & Cha, 2002). While these models replicate the end-stages of the disease, they do not characterise the progressive nature of the disorder and only reveal limited information about the pathogenic mechanisms underlying neurodegeneration. The principal advance that identification of the gene enabled was the development of genetic models of HD. Gene expression has been achieved in a range of hosts, from single cells through invertebrates to rodents. These models have been highly successful and have provided many key insights into the early stages of the disorder.

1.2.2 Symptoms of Huntington's disease

Huntington's disease is a progressive disorder that is usually associated with a midlife onset, generally characterised by a gradual loss of both voluntary and involuntary motor control. The most striking motor disorder is the choreic movements that can affect the trunk, head and limbs, causing uncoordinated flailing movements (figure 1.2.2).

As the disease progresses subcortical and, later, cortical dementias develop. Subcortical dementias arise as neurones within the basal ganglia, particularly the striatum, die. This gives rise to personality changes, reduced attention span and slowed thinking. As the cells within the cerebral cortex start to atrophy, cortical dementia also develops. Cortical dementia affects higher cognitive processes, such as planning, recall and recognition.

Following the identification of the gene, and the development of a genetic test for HD gene carriers, it has been realised that many patients present with dementia in the absence of motor deficits and that subtle cognitive changes can occur many years before classical symptoms and cell death are detectable. These subtle changes occur long before evidence of neuropathological changes, suggesting that events other than cell death underlie the early cognitive deficits. Given that alterations in the communication between neurones are believed to underlie learning and memory, it is possible that abnormalities in these processes could underpin the early deficits in cognition seen in HD.

1.2.2.1 "Presymptomatic" patients

The onset of Huntington's disease is generally viewed as the presence of the specific motor disorder (Kremer, 2002). However, following the isolation of the gene responsible for Huntington's disease (Huntington's Disease Collaborative Research Group, 1993), predictive testing has been developed and it has thus become possible to study gene carriers prior to the development of an overt motor disorder. It has become apparent that, long before the onset of classical symptoms and evidence of cell death, gene carriers often show some neurological changes, including altered brain function (see neuropathology, section 1.2.3) and a decline in cognition and motor control.

The motor abnormalities that are observed in preclinical patients are minor. In a seven year follow-up study of asymptomatic individuals, in which genetic status was confirmed using a marker known to be linked to HD, distinct abnormalities were identified. Abnormalities included general restlessness, hyperreflexia, excessive and inappropriate movements of the fingers, toes, hands and feet, abnormal eye movements and an impaired ability for finger tapping and these symptoms become particularly apparent during emotional stress (Penney *et al.*, 1990).

A range of cognitive parameters have been tested, with some reports highlighting deficits (discussed below), while others show no decline (for example see Campodonico *et al.*, 1996; Gomez-Tortosa *et al.*, 1996; de Boo *et al.*, 1997). The inconsistency in detecting early cognitive deficiencies may be due to small sample sizes, the age of the subjects (or rather their closeness to clinical presentation) and the sensitivity of the tests employed. Indeed, as the tests employed to assess cognitive

processing become more sensitive and specific, an increasing number of abnormalities have become apparent.

Early studies highlighted underperformance in tests to assess attention and planning skills (Rosenberg *et al.*, 1995), poor ability to sequence pictures and categorise objects (Foroud *et al.*, 1995; Lawrence *et al.*, 1998) and impairments in visual and verbal memory (Jason *et al.*, 1988; Hahn-Barma *et al.*, 1998). Others report a reduced reaction time to visual stimuli (Siemers *et al.*, 1996) although this may (arguably) be attributable to motor impairments rather than cognitive dysfunction. Emotional recognition is also affected, with gene carriers showing poor identification of faces displaying disgust, while showing good performance on background tests and identification of other facial expressions including happiness, sadness, surprise, anger and fear (Gray *et al.*, 1997).

Recognition memory

The article by Hahn-Barma *et al.* (1998) also highlighted significant differences between age-matched non-gene carriers and asymptomatic gene carriers in several parameters of the California verbal learning test. Parts of this test assess the ability to discriminate between novel and familiar items from two previously learnt shopping lists. Gene carriers show significantly less object recognitions and significant impairments in recognition discriminablity (an index of recognition accuracy that takes into account both misses and false positives).

More recently the significance of impairments in recognition memory was highlighted by a different test, that of signal detection. Signal detection provides a sliding scale of familiarity continuum, with "new" items at the lower end and "old"

items at the upper end. Variability in the familiarity of stimuli gives rise to two overlapping distributions for new and old cues and from these distributions a *d'* threshold can be obtained as an assessment for recognition impairment (see Yonelinas *et al.*, 1996 for a brief review). Individuals undergoing predictive testing at the Predictive Huntington's Disease Clinic at Addenbrooke's Hospital, Cambridge, U. K. underwent a wide range of psychiatric, cognitive and neurological tests designed to gauge the patients health, including neurological, personality deviance, depression, irritability, dissociation (failure to integrate thoughts, feelings and actions into consciousness) and a host of cognitive parameters. Following neurological and psychiatric testing, subjects were divided into gene positive and gene negative (control) groups. Based on CAG repeat length, age of onset was estimated according to the logarithmic algorithm proposed by Rubinsztein *et al.* (1997) and the mean age of the gene positive group was 13.8 ± 5.6 years below the estimated age of onset. It was found that recognition memory and increased irritability were the only significant deficits observed in otherwise asymptomatic gene-carriers compared to the age matched gene-negative group (Berrios *et al.*, 2002).

It appears, therefore, that impaired recognition memory is one of the earliest detectable deficits observed in Huntington's disease. It is also clear that the age of disease onset should not be considered to be the development of an overt motor disorder. Rather, the motor phenotype is a phase of Huntington's disease, which is often preceded by other symptoms, particularly cognitive deficits.

1.2.2.2 The "classical" symptoms

Although the classical symptoms of Huntington's disease usually present in midlife, the disease can occur at any age, with the youngest patient ever reported being two years of age, while at the other extreme, some patients do not present symptoms until their mid eighties (Roos *et al.*, 1991). The mean age of onset is usually considered as 40 years with a standard deviation of about 10 years, although this value may be misleading as the age of onset does not follow a normal distribution (see Kremer, 2002) and may be further confounded by sampling methods where late-onset cases may be excluded due to their lack of symptoms. Some studies have, therefore, only considered individuals that are considered "at risk" and are already deceased, thereby including all cases of HD, including those with exceptionally late-onsets. By considering only deceased patients, disease duration can also be assessed. One such study of 800 deceased Dutch patients gave a mean age of onset as late as 55.8 years with a duration of 15.6 years (Roos *et al.*, 1993).

In ninety percent of cases the most prominent symptom is chorea (see figure 1.2.2). Choreiform activity has been defined as 'excessive, spontaneous movements, irregularly timed, randomly distributed and abrupt' (Barbeau *et al.*, 1981). The severity of choreal movements can, however, vary from restlessness through an unstable dance-like gait to violent, uncontrolled movements. Choreic movements are continuously present in the awake state and cannot be effectively suppressed by the patient (Fish *et al.*, 1991). Chorea can affect various portions of the body. Facial chorea results in pouting of the lips, grimacing and twitching and may involve the neck, leading to head rotation and bending. Choreic activity of the trunk causes the

whole body to be moved about and can cause irregular breathing. Choreiform of the limbs leads to flexing of the fingers and toes; arms and legs may be constantly crossed and uncrossed (Kremer, 2002). The series of still photographs in figure 1.2.2 shows a patient with choreic movements.

As the disease progresses chorea tends to abate and a syndrome akin to Parkinson's disease develops. Bradykinesia and rigidity gradually occur and in some cases the patient becomes severely rigid (Young *et al.*, 1986; Penney *et al.*, 1990). Dystonia also develops in the later stages of HD (Young *et al.*, 1986) and is usually characterised by abnormal posturing of the extremities and the head. The prevalence of dystonia has been reported as high as 95 *per cent* (Kremer, 2002), however the similarity to chorea makes a distinction between the two movement disorders difficult.

The cognitive and psychiatric problems are very prominent and complex. Cognitive deficits are primarily of executive function i.e. the ability to plan, organise, and show mental flexibility. Furthermore, procedural memory and psychomotor functions are affected, whereas skills such as language are relatively spared (although speech is impaired as a result of motor dysfunction); for review see Craufurd and Snowden (2002). By the time patients reach moderately advanced stages of the disease there are clear deficiencies in visual and verbal recall and the deficits in recognition memory become more pronounced (Lange *et al.*, 1995; Lang *et al.*, 2000).

Psychiatric problems include dysphoria, euphoria, agitation, irritability, apathy, anxiety, disinhibition, delusions and hallucinations (Paulsen *et al.*, 2001), all of which can be very trying for both patients and their carers.

Figure 1.2.2: Chorea. The above set of stills shows a patient attempting to sit up. All her limbs show uncontrolled choreic movements. Images courtesy of Huntington's Dance Foundation.

1.2.2.3 Late- to end-stage

From the sufferer and his carer's point of view the late-stages of Huntington's disease are dominated by the increasing dependence on others for normal activities in day-to-day living and therefore patients are often cared for in nursing homes. Severe bradykinesia and rigidity result in decreased manual dexterity. Speech is often impaired leading to communication problems and swallowing becomes difficult causing problems with feeding (Kremer, 2002). Systemic disorders are very common, the most striking of which is weight loss, probably due to a decrease in dietary intake and possibly confounded by increased energy expenditure. There are often altered sleep patterns including reduced rapid eye movement and slow wave sleep, increased sleep onset latency and a reversal of day-night sleep patterns (Sishta *et al.*, 1974; Wiegand *et al.*, 1991; Kremer, 2002).

Cognitive assessment reveals a severe deterioration in all cognitive functions (Kremer, 2002). Severe psychiatric problems are often the cause of the patient's placement within nursing homes and require the use of psychotropic drugs, which in themselves can compound the problems of rigidity, gait and swallowing (Magnuson *et al.*, 2000; Stubner *et al.*, 2004). The extreme personality changes are particularly harsh on the patient's family and friends, particularly offspring who may be witnessing their own fate.

Following the onset of the "classical" symptoms of the disorder, patients generally live for a further 15 to 20 years (Roos *et al.*, 1993; Foroud *et al.*, 1999). Death, however, is not caused by Huntington's disease *per se*, but rather by associated complications. The most common causes of death of HD patients includes pneumonia (one third of all patients), heart failure (although heart disease, cerebrovascular disease

and atherosclerosis show no increase), choking and nutritional deficiencies (Lanska *et al.*, 1988). Suicide is an associated risk, with suicide rates of up to 7.3 *per cent* of all patient deaths; four times that of the general population (Di Maio *et al.*, 1993). This rather taboo subject is likely to be under-reported, with up to 27 *per cent* of possibly affected individuals attempting suicide at least once (Kremer, 2002). Likewise, suicide in the general population is likely to be underestimated; however, some reports have accounted for this and still find a significant increase in suicide rate amongst HD patients (for example see Schoenfeld *et al.*, 1984).

1.2.3 Neuropathology of HD

The classical hallmarks of Huntington's disease are believed to be attributable to neuronal cell loss within particular brain structures. For example, chorea is thought to be due to a loss of the GABAergic neurones of the motor pathway within the basal ganglia. The recently described early cognitive decline may be a consequence of subtle changes in neuronal function. For example, as discussed below, a loss of dopamine receptors has been closely correlated with the cognitive decline seen in asymptomatic patients and the loss of other neurochemical markers (e.g. other neurotransmitter receptors and neurotransmitter synthetic/degradatory enzymes) and subtle neuropathology may underlie these deficits.

1.2.3.1 The *post-mortem* HD brain

Examination of *post-mortem* brains from advanced HD patients reveals marked atrophy in selective areas (figure 1.2.3). Total brain weight is reduced by 10-20 *per cent* compared to age matched controls (Gutekunst *et al.*, 2002). In 1985 a grading system was established to rate the degree of atrophy (Vonsattel *et al.*, 1985). There are four Vonsattel grades (1-4), plus grade 0 (no cell loss). All grades show a loss in brain volume with up to 30% attributable to cell death. The basal ganglia show the greatest cell loss and the loss of this region is perhaps the most striking difference observed in HD post-mortem brain. The loss of cells within the striatum leads to enlargement of the ventricles and this is clearly seen in figure 1.2.3.

The basal ganglia are composed of the striatum (putamen and caudate), the subthalamic nucleus, the internal and external segments of the globus pallidus and the substantia nigra, which consists of the pars compacta and pars reticula (Graybiel, 1990, see figure 1.2.4). The primary excitatory input into the striatum derives from the cerebral cortex, which activates a doubly inhibitory striatopallidothalamic pathway mediated by γ-aminobutyric acid (GABA) containing neurones, constituting a positive feed-back loop. There is also an indirect pathway *via* the subthalamic nuclei that provides inhibition to the thalamus, thereby regulating this feed-back loop. This basic circuitry is further modulated by inputs arising from the substantia nigra and these are mediated by the neurotransmitter dopamine. Other modulators active within the pathway include acetylcholine (ACh), substance P, encephalin, somatostatin and neuropetide Y (Graybiel, 1990).

Figure 1.2.3: Neurodegeneration seen in the human brain as a result of Huntington's disease. The images on the left of this series are of *post-mortem* tissue from a 48 year old HD patient. The right hand images are from a 34 year old that did not suffer from HD. There is a general decrease in brain mass. Frank neurodegeneration can be seen in the striatum (S) evidenced by enlarged ventricles. Cortical (Ctx) atrophy is evident as a thinning of the cortical mantle and enlarged sulci. (Images adapted from Colb, 2003.)

The spiny GABAergic neurones are particularly susceptible to neuronal cell death, while the interneurones are relatively spared. The loss of neurones in this area goes hand-in-hand with the loss of neuronal markers. In particular the loss of medium spiny neurones in the striatum is marked by the loss of glutamic acid decarboxylase, substance P, calcineurin, calbindin, and adenosine, acetylcholine, 5-hydroxytryptamine (5-HT; serotonin) and dopamine receptors.

Within the striatum the density of the dopamine synthetic enzyme, tyrosine hydroxylase, is reported as unchanged. Given there is an overall loss of GABAergic neurones within this region, it would therefore appear that there is a down-regulation in dopaminergic levels (Yohrling IV & Cha, 2002). In support of this, the expression of the D_1 and D_2 subclasses of dopamine receptor are reduced in the HD brain, particularly within the striatum where they are located on the medium-sized spiny neurones (MSSNs, Reisine *et al.*, 1977; 1978).

Importantly, there is also a marked loss of cells within the cerebral cortex, with up to 33% reduction in neurone number (Vonsattel *et al.*, 1985; Heinsen *et al.*, 1994). As mentioned earlier, this is evident in the post-mortem brain, manifesting as thinning of the cortical mantle and increased invagination of the sulci (see figure 1.2.3). The pyramidal cell layers III and V are particularly affected (Braak & Braak, 1992; Gutekunst *et al.*, 2002) while the granular layer IV cells are relatively spared, leaving a conspicuous band between layers III and V (Heinsen *et al.*, 1994). This is particularly clear in the temporal, frontal and parietal lobes where preferential loss is seen of the layer III pyramidal cells.

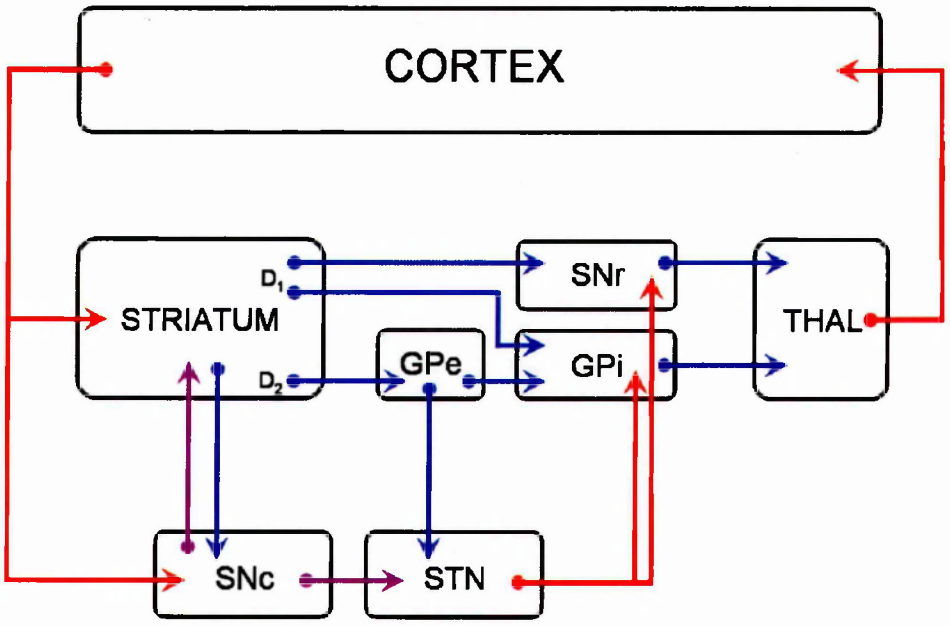


Figure 1.2.4: Neuronal circuitry of the basal ganglia. The nuclei that constitute the basal ganglia are interconnected in a complex network and are believed to be involved in motor coordination, cognitive function and subcortical processes. The striatum is the main input into the basal ganglia pathways and receives excitatory innervation from all areas of cortex, particularly the motor cortex. The direct pathway is composed of the double inhibitory striatopallidothalamic circuit and forms a positive feedback loop. The indirect pathway, *via* the subthalamic nuclei, provides an inhibitory regulation of the direct pathway. Dopaminergic modulation of the network derives from the substantia nigra. In Huntington's disease the GABAergic medium-sized spiny neurones are particularly susceptible to degeneration leading to loss of motor control. Abbreviations: Red arrow, glutamatergic neurone; Blue arrows, GABAergic neurone; Purple arrow, dopaminergic neurone; GPe, globus pallidus external segment; GPi, globus pallidus internal segment; SNr, substantia nigra pars reticulata; SNc, substantia nigra pars compacta; STN, subthalamic nucleus; THAL, thalamus. (Adapted from Schmidt, 1998.)

The loss of cortical neurones also goes hand-in-hand with a loss of neurochemical markers, although changes in neurochemical markers within the cerebral cortex is less characterised than in the striatum. In particular the level of the neurotransmitter GABA has been shown to be low. The neurotransmitters glutamate and GABA are reduced in the temporal and frontal cortices of the HD *post-mortem* brain (Pearson & Reynolds, 1994) while metabolites of dopamine are increased (Pearson & Reynolds, 1994). Furthermore, both glutamate and dopamine receptors are also reduced within the *post-mortem* HD cortical mantle (Yohrling IV & Cha, 2002).

Other affected areas include the hippocampus, particularly the CA1 field (Spargo *et al.*, 1993). The cerebellum, brainstem and spinal cord are generally considered as being spared, however this is only relative as degeneration is often observed within these brain regions (Gutekunst *et al.*, 2002).

Protein aggregates are commonly observed throughout the HD brain (DiFiglia *et al.*, 1997; Gutekunst *et al.*, 1999; Sieradzan *et al.*, 1999). They primarily contain fragments of the mutant protein responsible for Huntington's disease (see genetics, section 1.2.4; and Molecular biology, section 1.2.5). The precise role of aggregates in the pathogenesis of HD is still under debate, some arguing them to be neurotoxic and others as neuroprotective. Such aggregates are present in all areas of the brain, including the hippocampus and cerebellum, but are particularly high within the cortical mantle and corpus striatum.

1.2.3.2 Presymptomatic neuropathology

With the development of imaging techniques it has become possible to study neuropathological changes within the brain prior to death. Consistent with the impairments observed by cognitive testing, neuroimaging studies repeatedly demonstrate various deficiencies in the preclinical Huntington's disease brain. Positron emission tomography (PET) studies of asymptomatic HD gene carriers have demonstrated decreases in basal ganglia glucose metabolism (Antonini *et al.*, 1996), decreased dopamine receptor binding in both striatum (Antonini *et al.*, 1996; Andrews *et al.*, 1999; Weeks *et al.*, 1996; Pavese *et al.*, 2003) and cortex (Sedvall *et al.*, 1994; Pavese *et al.*, 2003; Andrews *et al.*, 1999). Cortical and striatal loss of dopamine receptors in an asymptomatic patient can be seen in figure 1.2.5. Structural magnetic resonance imaging has also revealed a decrease in basal ganglia volume many years before the predicted age of onset (Aylward *et al.*, 1996).

The decline in dopamine receptor expression parallels the decreased detection of mRNA for D_1 and D_2 in patients in the early stages of HD (Vonsattel grades 0 and 1, Augood *et al.*, 1997). Moreover, the reduction in D_1 and D_2 dopamine receptors in "asymptomatic" gene-carriers correlates well with the learning deficits observed upon cognitive testing (Bäckman & Farde, 2001).

In light of the changes observed in the brains of gene carriers prior to the onset of classical symptoms, it seems likely that the preclinical cognitive deficits are indeed real and may be seated in the loss of neurochemical markers.

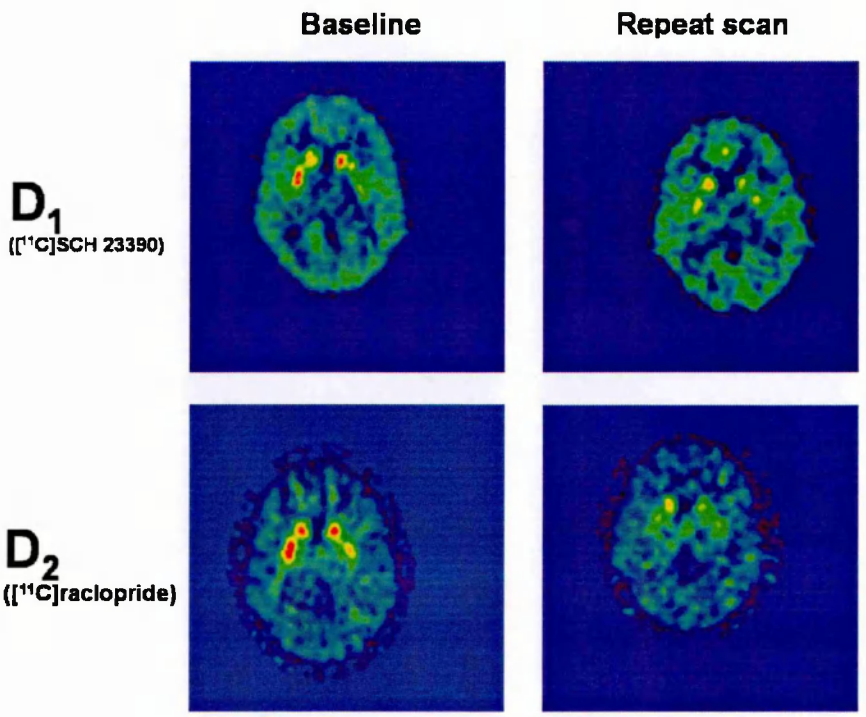


Figure 1.2.5: PET scans demonstrating a progressive loss of dopamine receptors in an asymptomatic patient. Baseline and repeat [^{11}C]SCH23390[1] (D_1) and [^{11}C]raclopride[2] (D_2) PET binding potential maps in an asymptomatic individual carrying an expanded CAG repeat in the HD gene. Scans were performed at an interval of 33 months. Loss of both D_1 and D_2 binding is apparent in both the basal ganglia and cortex. (Adapted from Andrews *et al.*, 1999.)

[1] SCH23390 is a D_1 dopamine receptor antagonist
[2] Raclopride is a D_2 dopamine receptor antagonist

1.2.3.3 Neuropathology of symptomatic individuals

As clinical symptoms begin to manifest the changes within the brain become more apparent. These neuropathological changes are strongly correlated with both the motor and cognitive decline observed at this stage of disease progress. In particular, the loss of motor control is may be caused by the loss of the spiny GABA containing neurones within the striatum, which constitute the motor pathway. Loss of inhibition within this pathway leads to inappropriate excitatory outputs and hence decline in motor control.

Anatomical changes of neuronal processes are frequently seen within the HD brain. Dendritic remodelling is quite dramatic, even before overt cell death. Dendrites undergo truncation and swelling and there is a marked loss of spines (Sotrel *et al.*, 1993; Gutekunst *et al.*, 1999). Axonal projections become condensed, convoluted and fragmented with dystrophic cortical neurites present, particularly in the cortical pyramidal layers III and V (Jackson *et al.*, 1995).

Aggregates become apparent at this stage, appearing first within the cortex and latter in the striatum and hippocampus. Within the cortex aggregates are commonly observed in the neuropil. More specifically, within layer III of the cortex, 60-100% of aggregates are cytoplasmic, while in layer V up to 65% are located within the nucleus. Inclusions within the striatum are most regularly found as nuclear aggregates (Gutekunst *et al.*, 1999).

Changes in neurotransmitter receptor binding and blood flow are observed, as measured by PET and single photon emission computerised tomography (SPECT) respectively (for review see Brooks & Andrews, 2002). Several PET studies reveal

marked reductions in agonist binding to both D_1 and D_2 dopamine receptors in striatum, amygdala and temporal and frontal cortices (Ginovart *et al.*, 1997; Andrews *et al.*, 1999; Pavese *et al.*, 2003). Further evidence for abnormal cortical function comes from measurements of blood flow using SPECT during the performance of tests designed to assess various aspects of cognition. Impairments in tests to assess language, perception and word recognition strongly correlate with a decrease in cortical blood flow (Hasselbalch *et al.*, 1992).

Taken together these data suggest that an array of changes may take place within the cerebral cortex during HD and that the resulting cortical dysfunction may underlie the deficits in cognitive processes associated with the cerebrum.

1.2.3.4 Pathogenic mechanisms

Several mechanisms of pathogenesis have been proposed to play roles in Huntington's disease, including excitotoxicity and oxidative stress. It has also been proposed that the presence of aggregates could be causative of neuronal cell death. These mechanisms will be described in more detail in later sections of this chapter.

Excitotoxicity

Inappropriate activity of excitatory neurotransmitter systems have been implicated in the pathology observed in HD. Evidence to support this theory has come mostly from animal models, where administration of neurotransmitter analogues have been shown to mimic the pattern of cell loss observed in HD (see Animal models, section

1.2.7). Excitotoxicity is thought to be mediated by altered neurotransmitter receptor activity, resulting in elevated calcium levels within the cell, which is known to induce cell swelling and subsequent cell death (see Garthwaite, 1994 for review).

Oxidative stress

Evidence that oxidative stress could play a role in HD come from the identification of increased levels of the oxidative damage products malondialdehyde, 8-hydroxydeoxyguanosine, 3-nitrotyrosine and heme oxygenase in nuclear DNA within brain regions particularly affected in HD (Browne *et al.*, 1997; 1999). The presence of these epitopes could, however, be either the cause of cellular death or be a by-product of the cell death cascade.

Aggregation

Many have proposed that the build up of mutant protein could be the cause of atrophy. The basis of this idea is that they pre-date cellular loss. In contrast, their distribution does not reflect the pattern of cell loss observed in HD. For example aggregates appear first in the cortex, while the greatest cell loss is within the striatum; aggregates are common within Purkinje cells of the cerebellum, but this region is relatively spared. It has therefore been argued that aggregation is a protective mechanism allowing the toxic protein to be removed from the system following failure of other mechanisms (see Molecular biology, section 1.2.5).

Apoptosis and necrosis

The process of cell death usually occurs by one of two processes: Apoptosis or necrosis. Apoptosis is characterised by pyknosis, chromatin condensation, membrane blebbing and formation of apoptotic bodies. Necrosis, on the other hand, is associated with cell and organelle swelling, lysis and other inflammatory responses. In the human condition little is known about the mechanism by which cell death occurs and the neurodegeneration seen in the mouse models fits neither of these mechanisms well. This alternative mechanism of cell death was identified in the R6/2 transgenic mouse model of HD (see section 1.2.7) and has been termed "dark cell degeneration" due to an enhanced affinity for staining by either toluidine blue or osmium (Turmaine *et al.*, 2000). The neurones show condensation of both the nuclear and cytoplasmic compartments and a clumping of chromatin, but the subcellular organelles appear intact. During the process of neurodegeneration, however, organelles, including the mitochondria and Golgi complex, do undergo a transient swelling and then condensation. The plasma membrane appears ruffled (Turmaine *et al.*, 2000). All the degenerating cells identified contained at least one nuclear inclusion. Dark cell degeneration was primarily seen in all cell layers of the anterior cingulate cortex from approximately 14-14.5 weeks of age. From approximately the same age (14-15 weeks), but to a lesser degree, dark cell degeneration was also detectable within the anterior striatum and the Purkinji cell layer of the cerebellum, and from 16 weeks within the lateral areas of the frontal cortex (Turmaine *et al.*, 2000). Importantly, similar dark cell degeneration was identified by the same authors within the same brain regions of *post-mortem* HD brain tissue (Turmaine *et al.*, 2000). Furthermore, similar degeneration is seen in

another transgenic HD model, the *drosophila* (see section 1.2.7). Degenerating neurones within the eye were characterised by enhanced staining with osmium, show condensation of the cytoplasm and nucleus, contain intranuclear inclusions, but do not show signs of apoptosis (Jackson *et al.*, 1998). An example of the neurodegeneration seen in the drosophila is shown in figure 1.2.13.

1.2.3.5 Treatment

Despite the wealth of knowledge of many of the processes involved in the progress of the disease, treatment is still limited to symptom relief; no cure is available. In this manner, little has changed since the nineteenth century, although the range and quality of treatments available to treat the symptoms has improved. Treatment is targeted to improve the day-to-day life of the patient by reducing the motor and psychiatric abnormalities. In light of the neuropathological changes seen within the brain of HD patients, various approaches to treatment for chorea have been taken. Previously, attempts have been made to either increase or decrease the levels of GABA and dopamine within the basal ganglia. The success of these therapeutic approaches have varied greatly, with some reports of improvements in both motor and psychiatric aspects, while others report no change, or even that such treatment compounds the symptoms (for examples see Loeb *et al.*, 1976; Corsini *et al.*, 1978; Bartholini & Lloyd, 1980; Tsuneizumi *et al.*, 1994; Tedroff *et al.*, 1999).

More recently it has been proposed that neuronal transplants may be of benefit to HD patients. Initial studies have reported some success and may have

potential role in treatment (for example see Bachoud-Lévi *et al.*, 2000a; Freeman *et al.*, 2000a; Gaura *et al.*, 2004).

Interestingly, evidence from one of the mouse models suggests that environmental enrichment can delay the onset of disease (van Dellen *et al.*, 2000). In light of this, comprehensive occupational therapy may be of benefit to known gene carriers and to patients in the early stages of the disease.

Dopaminergic strategies

Attempts to reduce choreic movements using dopaminergic strategies are based on the theory that depletion of dopamine within the basal ganglia should reduce nigrostriatal dopaminergic transmission and hence inhibition of the indirect motor pathway (van Vugt & Roos., 1999). Reserpine is an example of a monoamine depleting agent (depletes all monoamines, including dopamine, at all presynaptic terminals of the brain) that has been employed in suppressing chorea. Several studies have reported a reduction in involuntary movements (summarised in van Vugt & Roos., 1999), however a decline in manual dexterity has been reported with this drug (Oliphant *et al.*, 1960). Furthermore, extrapyramidal effects, including depression, are associated with this treatment, which limits its use in HD.

Similarly, antipsychotic (neuroleptic) drugs have frequently been used in the treatment of chorea and, to a degree, have helped suppress these unwanted movements. In particular the dopamine receptor antagonists chlorpromazine, haloperidol, sulpiride and pimozide are commonly employed. Haloperidol is perhaps the most successful of these drugs and, while the precise pharmacological action of this drug is not fully understood, it would appear to involve the blockade of the D_2

subclass of dopamine receptor (see van Vugt & Roos., 1999). Chronic administration of haloperidol in primates has been associated with an up-regulation of D_2 dopamine receptors and a down-regulation of D_1 (Lidow & Goldman-Rakic, 1994). This change in the expression levels of dopamine receptor subclasses could explain the depression associated with long-term use of this drug.

GABAergic strategies

Attempts to increase GABA levels have met with less success. Administration of GABA precursors, GABA itself, GABA receptor agonists or inhibitors of GABA transaminase have all been tried but have rarely been reported as beneficial to HD patients (see van Vugt & Roos., 1999). The antichoreic effects observed can often be explained by the sedative side effects of these substances.

The management of the wide variety of psychological and psychiatric problems includes both medicine and a comprehensive counselling regime. The success of these regimes is, however, often only anecdotal and vary immensely. Common drugs in use are atypical antidepressants and the 5-HT re-uptake inhibitors (Nance & Westphal, 2002).

While the motor and psychological aspects of HD are often treated (if not completely successfully), attempts to improve cognition are generally overlooked.

Transplants

Given that the striatum shows the greatest degree of atrophy, attempts have been made at transplanting neural stem-cells into the striatum. Initial studies were carried out in models of HD created by inducing striatal lesions in rats (see section 1.2.7) (for example see Wictorin & Bjorklund, 1989; Wictorin *et al.*, 1989; Pundt *et al.*, 1996). It was shown that transplanted striatal neuroblasts (foetal neurones) integrate into the host brain and even ameliorates the locomotor deficits induced by the striatal lesion. Similar improvements were shown in primate lesion models following transplant (for example see Hantraye *et al.*, 1992b; Kendall *et al.*, 1998; Palfi *et al.*, 1998). Moreover, striatal transplants into human HD patients have been shown to be successful. One study followed a patient for 18 months following transplant (the patient died of coronary associated problems). Upon post-mortem examination it was found that, like normal striatal cells, the grafted cells received topographically organised cortical afferents and had established appropriate efferents to globus pallidus and substantia nigra pars reticularta. Furthermore, selective markers for both striatal projection and interneurones (for example dopamine and cyclic 3',5' adenosine monophosphate (cAMP)-related phosphoproteins, acetylcholine acetyltransferase, tyrosine hydroxylase, calbindin, encephalin and substance P) showed areas of positive transplants that were innervated by host tyrosine hydroxylase fibres. Lastly it was noted that grafted neuronal cells did not develop inclusions, nor was there any sign of degeneration (Freeman *et al.*, 2000b).

In another report, PET was used to assess metabolic activity within the striatum of HD patients following striatal transplant of foetal cells. In three of the five patients studied an increase in metabolic activity was observed that correlated with motor and cognitive improvements (Bachoud-Lévi *et al.*, 2000b). More recently the same research group has reported that striatal grafting also improves metabolic activity within the motor cortex, suggesting that the whole corticostriatal loop is improved (Gaura *et al.*, 2004).

Despite the improvement in day-to-day life resulting from striatal grafts of foetal neurones, such therapy can not aid improvement to areas outside of the corticostriatal loop, nor provide a complete cure and therefore drug-based therapies are (and probably will remain) necessary.

1.2.4 The genetics of Huntington's disease

The hereditary nature of this form of chorea was one of the main traits that caught George Huntington's attention. Many studies have since been carried out looking at family pedigrees and patterns of inheritance. In particular families in the Zulia region of Venezuela have been studied extensively, with over 10, 000 members listed and over 100 living affected subjects, all derived from a single founder. A full account of the project is not available (touched upon in Wexler, 1996), but this project has proved invaluable, providing subjects for the gene mapping which was finally achieved in 1993 (see below). HD is inherited in an autosomal dominant fashion and appears to follow standard Mendelian rules, with parental transmission fitting closely to the expected 50 *per cent* (Harper & Jones, 2002, see figure 1.2.6). Families that have both parents carrying the HD gene have extra considerations, as three out of four offspring will develop the disease, one of whom is likely to be homozygous. All offspring from homozygous individuals, irrespective of their partner's genotype, will carry the gene (see figure 1.2.6).

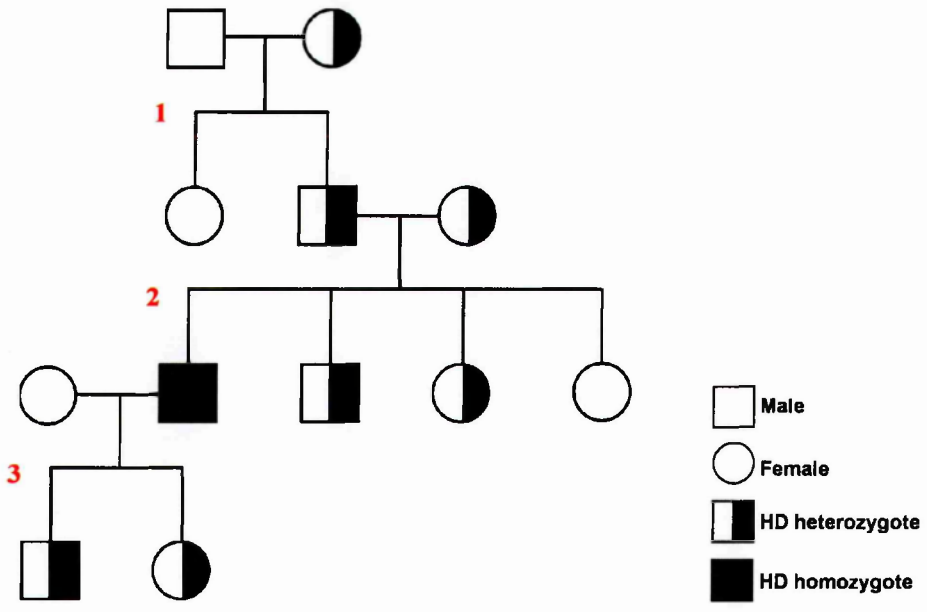


Figure 1.2.6: Pedigree showing typical Mendelian inheritance of HD. When one parent is affected offspring have a 50% chance of inheriting the disease (1). If both parents are heterozygous for the gene 75% of offspring are likely be affected; one in four will be homozygous (2). All offspring from a homozygous individual, irrespective of their partner's status, will inherit the abnormal gene (3). The sex of an individual is not important.

1.2.4.1 Identification of the gene

Mapping of the gene started in the early 1970s (Lindstrom *et al.*, 1973) and the first confirmed linkage of Huntington's disease came in 1983 when the gene responsible was located to the short arm (p) of chromosome 4 by using a DNA probe (G8), known to be linked to Huntington's disease (Gusella *et al.*, 1983). It took another ten years before the actual gene was mapped and found to contain an expanded trinucleotide repeat (Huntington's Disease Collaborative Research Group, 1993). The gene (which is presented in a simplified form in figure 1.2.7) was termed '*interesting transcript 15*' (*IT15*), contains 67 exons and encodes a large protein of approximately 348 kDa which has been termed huntingtin. Near to the *N*-terminal and located within exon 1, is a trinucleotide repeat of cytosine-adenine-guanine (CAG), which, following translation, encodes a tract of the amino acid glutamine (Q). Following the CAG repeat there is a CAA codon followed by another CAG. As CAA also encodes glutamine the polyglutamine stretch is actually two Qs longer than the initial CAG number (Huntington's Disease Collaborative Research Group, 1993); this often causes confusion in reports of repeat length (Gellera *et al.*, 1996).

Huntington's disease is one in a growing list of diseases caused by expansions in polyglutamine repeats. The list includes spinal and bulbar muscular dystrophy (SBMA, also known as Kennedy's disease, La Spada *et al.*, 1991), dentatorubral-pallidoluysian atrophy (DRBLA, Koide *et al.*, 1994) and several variants of spinocerebellar ataxia (SCA), namely type 1 (Orr *et al.*, 1993), type 2 (Imbert *et al.*, 1996), type 3 (also known as Machado-Joseph disease, Kawaguchi *et al.*, 1994), type 6 (Zhuchenko *et al.*, 1997), type 7 (David *et al.*, 1997) and type 17

(Koide *et al.*, 1999). These diseases present with a wide range of symptoms, but also have many in common. They often have a midlife onset, are progressive neurodegenerative diseases and have complex motor, cognitive and psychiatric components (Bates & Benn, 2002). Identifying how the CAG repeat expansion bestows toxicity in one disease may aid developments in the other polyQ disorders.

1.2.4.2 CAG repeat length

Development of Huntington's disease is highly CAG repeat length-dependent. In the normal population, the CAG repeat is of between 7 and 35 repeats. Individuals carrying more than 35 repeats will, however, go on to develop the disease at some point within their lifetime (Andrew *et al.*, 1993; Duyao *et al.*, 1993; Snell *et al.*, 1993). The age of onset (and to a degree the severity of the disease) and hence the age at death, are inversely correlated with the length of the expanded CAG repeat, such that those with longer repeats develop the disease earlier. Individuals with greater than approximately 60 CAG repeats often develop juvenile Huntington's disease (Harper, 1999). The relationship between CAG repeat length and age of onset can be clearly seen in figure 1.2.7 and is summarised in table 1.2.1.

There is a large variation in age of onset for any given CAG repeat length within the intermediate range (35-50 CAGs). For example, a repeat length of 40 CAGs leads to an onset ranging from 40 to 70 years of age (North American and Canadian population, figure 1.2.7). This variation means that, although logarithmic algorithms have been proposed for predicting the age of onset (for example see

Rubinsztein *et al.*, 1997), in reality predicting the precise age at which clinical signs will manifest is not practical.

The correlation between polyQ length and clinical symptoms is harder to dissect because of the overriding age of onset correlation. The clearest correlation is shown by juvenile onset HD, consequential of an extremely large CAG expansion, resulting in not only an earlier onset but also far more aggressive symptoms including rigidity and seizures (see next section). A correlation between psychiatric symptoms and repeat length has failed to be shown in several reports (see MacMillan *et al.*, 1993; Claes *et al.*, 1995; Weigell-Weber *et al.*, 1996; Zappacosta *et al.*, 1996). There does appear, however, to be a correlation between delayed visual reaction times and CAG repeat in "pre-symptomatic" gene-carriers (Siemers *et al.*, 1996). These patients also show delayed decision making. Other early cognitive impairments have been correlated with CAG repeat, particularly in other assessments of visual perception and verbal recall (Foroud *et al.*, 1995). It would therefore appear that CAG repeat length, while playing a role in early events, does not influence the severity of the clinical symptoms.

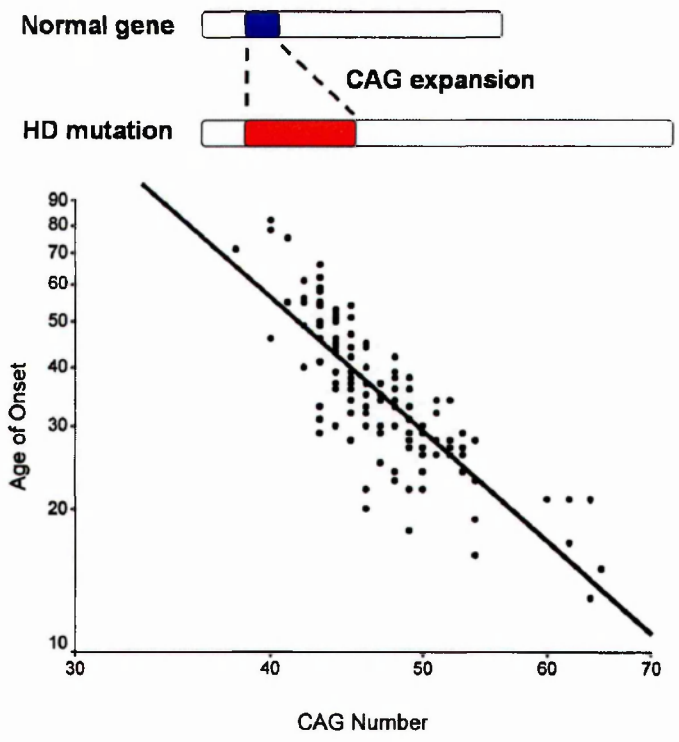


Figure 1.2.7: CAG repeat expansion and age of onset. *Top* A representation of the gene responsible for Huntington's disease. In the normal population the gene carries a CAG repeat of up to 35 near to the *N*-terminal. Individuals with HD have an expanded CAG repeat of greater than 35. *Bottom* Age of disease onset is highly correlated with CAG repeat length. Each point corresponds to an individual with Huntington's disease. Best fit line is represented. Axes represents CAG repeat number and age of onset in years, using a log-log scale. (Adapted from Rosenblatt *et al.*, 2001.)

$(CAG)_n$	Disease status
<28	Normal
29-34	Normal, but next generation at risk
35-40	Disease likely, offspring at high risk
41-85	Will develop the disease, 50% of offspring also at risk
>85	Juvenile onset

Table 1.2.1: CAG repeat length determines disease status.

Similarly, there is no evidence to support the theory that polyQ repeat length influences the rate of disease progression, although this may, again, be complicated by the age of onset (Harper & Jones, 2002).

Pathological studies have shown a correlation between CAG repeat length and cell death. One study shows a linear correlation between the degree of striatal atrophy at death with the CAG repeat length (Penney *et al.*, 1997). Other studies have demonstrated the number of inclusions in post-mortem brain are also correlated with CAG repeat (Becher *et al.*, 1998; Sieradzan *et al.*, 1999).

1.2.4.3 Juvenile HD

Soon after George Huntington's description of the disease it was realised that a juvenile form also occurs. One of the most detailed cases is that of a three generation HD family with daughters that presented with a severe form of the disease at the ages of four and ten. Symptoms included rigidity, hypokinesia, seizures and choreic movements (Hoffmann, 1888). This form of the disease is often referred to as the 'Westphal variant', although Westphal attributed these symptoms to a cause other than HD (Westphal, 1883). Juvenile cases account for approximately one *per cent* of all HD patients and two *per cent* of these show symptoms before the age of ten years (Rasmussen *et al.*, 2000). The youngest case presenting clinical signs was two years old (Osborne *et al.*, 1982). As mentioned earlier, juvenile HD is caused by extremely long glutamine repeats, generally over 50 in length (Kremer, 2002) although individuals with as many as 250 have been reported (Nance *et al.*, 1999). The first indications of juvenile HD are often poor performance at school, coupled

with behavioural disorders and possibly schizophrenic-like problems (Woldag *et al.*, 1997). As the disease develops a full, progressive dementia becomes apparent. Chorea is present in most cases, but is often mild and short-lived, soon replaced by rigidity, bradykinesia, hyperreflexia and other motor disturbances. Tonic-clonic and absence seizures are extremely common, occurring in up to 50% of cases (Hayden *et al.*, 1981; Osborne *et al.*, 1982).

While giving rise to an exceptionally aggressive form of the disease, the extremely long CAG repeats have been utilised in producing many of the genetic models of HD discussed in section 1.2.7.

1.2.4.4 Repeat instability

The number of CAG repeats can be unstable between generations. Interestingly the majority of juvenile cases arise from paternal transmission. This was confirmed in a study of seven families with 14 affected children and these data were complemented with a review of over 100 previously reported cases (Merrit *et al.*, 1969). The sex of the affected offspring was approximately 50:50. In contrast 80% of transmissions were from the father. Another intriguing observation is that successive generations of paternally transmitted cases show progressively earlier onsets (Newcombe *et al.*, 1981), a phenomenon known as *anticipation*.

The cause of anticipation is a sex-specific increase in CAG repeat length; large repeat expansions of larger than seven are almost exclusively seen in males (21% of all male transmissions versus 0.7% of all female transmission, Kremer *et al.*, 1995). Anticipation is thought to occur more in males than females due to the

differences in the process of gametogenesis between the sexes. In males, sperm is produced throughout sexual maturity by meiosis. In contrast, the dogma of oocyte generation in females, is that all meiotic events occur prenatally and thus a full complement is laid down before birth (Zuckerman, 1951). Therefore the frequency of meiotic events and the duration over which they take place is far greater in males than in females. It would appear CAG repeats are particularly unstable during meiotic division. Indeed, the size of repeat lengths in sperm shows a greater repeat length than other tissues from the same patient (Andrew *et al.*, 1993; Huntington's Disease Collaborative Research Group, 1993) and an increased variability (Leeflang *et al.*, 1995). Interestingly it has recently been shown that meiotic events may occur throughout female sexual maturity in order to maintain oocyte number (Johnson *et al.*, 2004). The incidence of meiotic events in females still remains low compared to males and this could explain the differences observed in the incidence of maternal and paternal anticipation.

Repeat lengths in the normal range appear to be stable. Very few, if any, truly spontaneous HD cases have been reported. Early studies estimated that isolated cases represent approximately 5% of all HD cases. Following more rigorous criteria this value falls to just 0.04% (see Harper & Jones, 2002). Since the isolation of the gene, it has become apparent that the isolated cases arise in transmissions where the parents have repeat lengths at the upper end of the normal region (33-36 CAGs, Huntington's Disease Collaborative Research Group, 1993, see table 1.2.1). It would appear, therefore, that there is an intermediate range in which an individual is not likely to develop the disease, but offspring are at risk of inheriting an expanded CAG repeat and thereby be at risk.

1.2.4.5 Huntingtin expression

Wild-type huntingtin is ubiquitously expressed in all tissues, with highest levels within the brain (Sharp *et al.*, 1995). Within the brain all regions, including cerebral cortex, striatum, globus pallidus, cerebellar cortex, hippocampus, amygdala and substantia nigra, express the protein (Landwehrmeyer *et al.*, 1995; Sapp *et al.*, 1997). Within the cerebral cortex, huntingtin expression is particularly high in cell layers II-IV and VI (Landwehrmeyer *et al.*, 1995). Expression is almost exclusively cytoplasmic, with higher levels within the soma than in the dendrites (Sapp *et al.*, 1997). Huntingtin is also particularly concentrated within nerve terminals (Sharp *et al.*, 1995).

In the HD brain, huntingtin is expressed in all regions and, with the exception of the putamen and globus pallidus, is expressed at normal levels. Within the putamen a significant increase in expression is seen, while in the pallidus region huntingtin is reduced (Landwehrmeyer *et al.*, 1995). At the cellular level, huntingtin expression within the HD brain is altered. There is an increased level of staining within the perinuclear region and a reduced labelling in neuronal processes (Sapp *et al.*, 1997). Moreover, there is intense staining of huntingtin within the nucleus, which is absent in control brains. Furthermore there is punctate staining both within the neuropil and soma.

1.2.4.6 Mosaicism

As suggested in the above text, different tissues within the same individual may contain CAG repeats of various lengths, with sperm carrying the highest repeat lengths. Mosaicism has also been reported in most somatic tissues, particularly the brain. Cells within the striatum and cerebrum show the largest increases in repeat number (Telenius *et al.*, 1994; Aronin *et al.*, 1995) and a similar pattern of mosaicism has been reported in some mouse models of HD (Mangiarini *et al.*, 1997; Wheeler *et al.*, 1999; Kennedy & Shelbourne, 2000).

The precise mechanism by which repeat expansion occurs is unclear, but could involve DNA replication, recombination, transcription or repair (see Jones, 2002).

1.2.5 Molecular biology of mutant huntingtin

Given the strong correlation between CAG repeat length and the age of onset (and to a degree the severity of disease) it is clear that the toxic nature of mutant huntingtin derives from the expanded glutamine stretch. A common feature of mutant huntingtin is its propensity to form insoluble aggregates, both *in vivo* and *in vitro*. How the aggregation of mutant protein confers selective neurotoxicity is unclear. Conversely it has been argued that aggregation could be a neuroprotective mechanism where by the toxic mutant huntingtin is removed from the system thereby defending neurones against mutant huntingtin-induced cell death (for example see Saudou *et al.*, 1998).

1.2.5.1 Aggregation

The idea that protein aggregation could be a key turning point in disease progression came in 1997 when it was reported that mice transgenic for exon 1 of the human gene develop inclusions within the nucleus of striatal, cortical, hippocampal, cerebellar and spinal cord cells before the onset of a neurological phenotype (Davies *et al.*, 1997; Scherzinger *et al.*, 1997). The inclusions were immunopositive for both huntingtin and ubiquitin. They were never seen in brains of non-transgenic mice. Similar inclusions had been reported previously in biopsy tissue from human cases (Roizin *et al.*, 1979) but largely ignored because of their apparent absence in post-mortem tissue (Forno & Norville, 1979). Credence for neuronal intranuclear inclusions came later when it was found that human patients do develop inclusions (DiFiglia *et al.*, 1997), a finding that has since been corroborated (Gutekunst *et al.*, 1999; Sieradzan *et al.*, 1999). As discussed in the animal models section (1.2.7), aggregates have also been observed in many transgenic models of HD.

The idea that large stretches of glutamines could lead to covalent cross-linking was proposed in 1993. In this model it was suggested that when a number of glutamine repeats exceeds a certain threshold, a protein (huntingtin or any other polyQ-containing protein) could become a transglutaminase substrate (Green, 1993). Transglutaminases catalyse the formation of insoluble, cross-linked proteins by way of covalent bonds (Greenberg *et al.*, 1991) and have been shown to be elevated in HD brains (Karpuj *et al.*, 1999). In light of both this suggestion and the CAG repeat length-dependency of HD onset (Huntington's Disease Collaborative Research Group, 1993), an atomic model of aggregation was formed (Perutz *et al.*, 1994). The model, known as the 'polar-zipper' model, proposes that the glutamine stretch forms

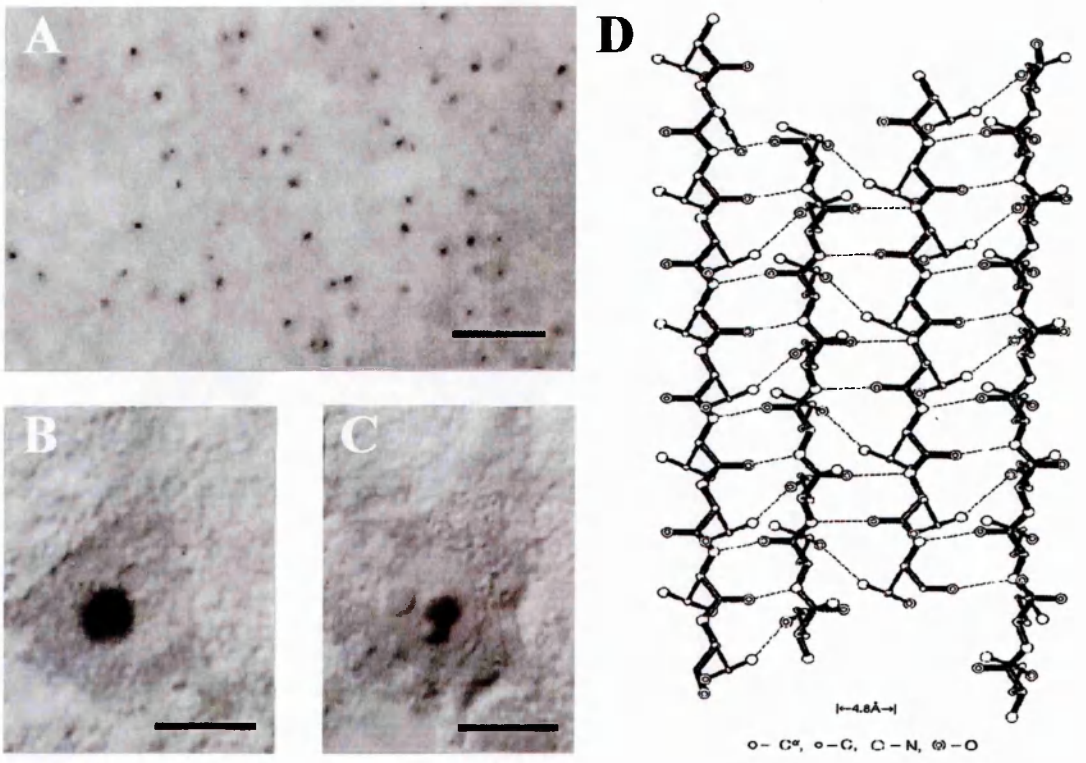


Figure 1.2.8: Neuronal intranuclear inclusions. A-C: Huntingtin immunoreactivity in the cortex of a juvenile HD patient. Examples of single (B) and double (C) inclusions in cortical pyramidal neurones. A, bar = 50 μm; B & C, bar = 10 μm. (Adapted from DiFiglia *et al.*, 1997.) D: Proposed inclusion structure. Expanded glutamine repeats lead to the formation of β-sheets, linked together by hydrogen bonds (dashed lines). (From Perutz *et al.*, 1994.)

antiparallel β-sheets (Perutz *et al.*, 1993). The hairpins that form then associate together and precipitate the protein. The polar-zipper model is shown in figure 1.2.8. It appears that aggregated huntingtin sequesters other proteins into the inclusion. In particular, transcription factors, caspases, protein kinases, parts of the proteasome and wild-type huntingtin have been identified within inclusions (see Jones, 2002 for review). Requisition of other proteins into huntingtin aggregates results in loss of function of those proteins and thereby affects their cellular processes.

1.2.5.2 Cleavage

Interestingly, in human brains, aggregates were only detectable by antibodies raised to the *N*-terminus, but not the *C*-terminus, of huntingtin (DiFiglia *et al.*, 1997). This suggested that accumulation was of truncated segments rather than full length protein. Indeed it has been found that mutant huntingtin is highly susceptible to proteolysis, which has been shown to be mediated by caspases (Wellington *et al.*, 2002) and calpains (Gafni & Ellerby, 2002).

Caspases are a family of proteolytic enzymes that are so named for having cysteine (C) residues at their active sites and cleave after aspartic acid (Asp) residues in their substrate proteins (Cooper, 2000). Huntingtin was the first neuronal protein to be identified as a substrate for caspase activity (Goldberg *et al.*, 1996) and, importantly, has been shown to have defined sites for caspases 2, 3 and 6 near to the amino-terminal portion (see Wellington *et al.*, 2000; Wellington *et al.*, 2002, see figure 1.2.9). Also, caspase-1 expression is increased in a mouse model of HD (Li *et al.*, 2000b) and inhibition of caspase-1 activity by mutation of the *caspase-1* gene

within R6/2 mice (see section 1.2.7), delays the onset of inclusion formation, prolongs survival and prevents the reduction observed in dopamine receptor binding within these mice (Ona *et al.*, 1999). Although there are viability questions associated with double transgenics, this would suggest caspases play an important role in the development of the pathogenesis seen in HD.

Similarly, calpains are a group of proteases that are expressed intracellularly, are calcium-dependent and may be involved in apoptotic processes. Calpain activation is detected in human HD tissue, but not in age-matched controls. Moreover, they appear to cleave mutant huntingtin in a CAG-repeat length-dependent manner into three *N*-terminal cleavage products (Gafni & Ellerby, 2002, see figure 1.2.9).

The finding that huntingtin appears to aggregate within the nucleus is interesting. Huntingtin does not appear to have a nuclear localisation signal, which is generally required for nuclear localisation of proteins (Xia *et al.*, 2003). In contrast, huntingtin does have a nuclear export signal, which is located near to the carboxy terminal (Xia *et al.*, 2003, see figure 1.2.9). The presence of the nuclear export signal should confer a cytoplasmic expression of huntingtin and, indeed, this is the case for wild-type protein (Sharp *et al.*, 1995; Xia *et al.*, 2003). Following cleavage by caspases and/or calpains, the nuclear export signal is no longer associated with the *N*-terminal fragment, allowing nuclear entry of the protein containing expanded polyQ tracts (Xia *et al.*, 2003) and subsequent inclusion formation.

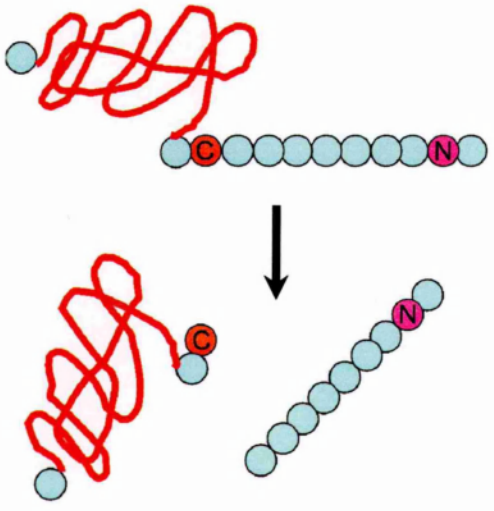


Figure 1.2.9: Cleavage of mutant huntingtin. The huntingtin protein carrying an expanded glutamine tract is represented. Cleavage at the caspase/calpain site (C) leads to dissociation of the *N*-terminal fragment from the nuclear export signal (N) contained in the *C*-terminal fragment. The *N*-terminal fragment can then enter the nucleus where it disrupts transcription and forms aggregates. (Based on data in Wellington *et al.*, 2000; Wellington *et al.* 2002; Gafni & Ellerby, 2002; Xia *et al.* 2003.)

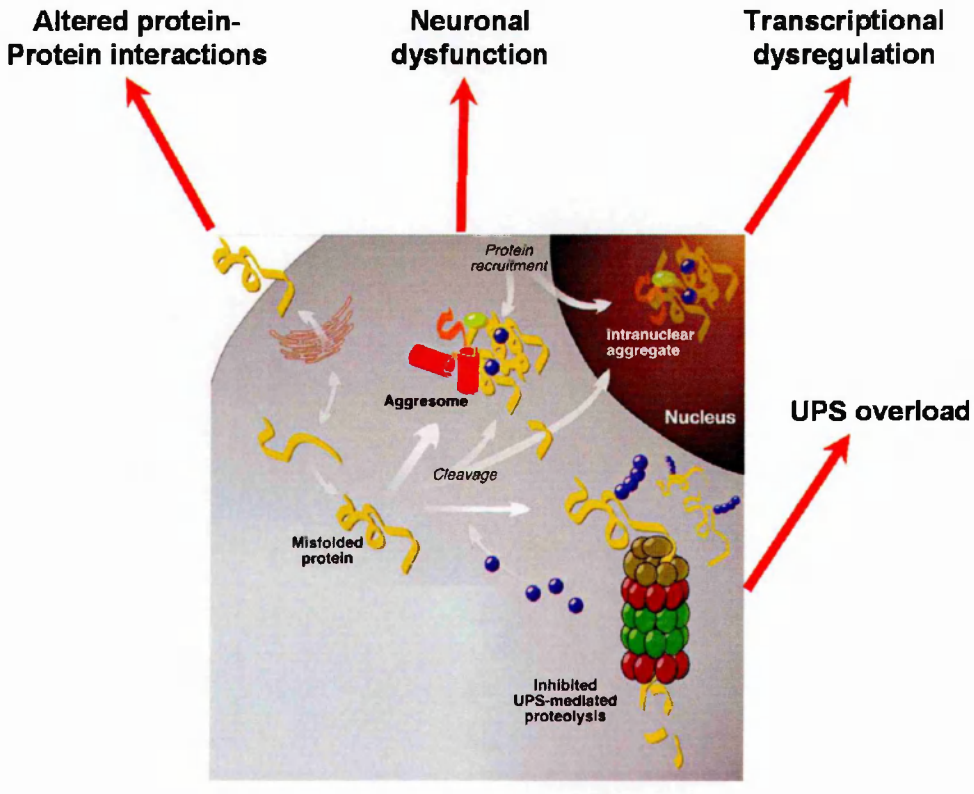


Figure 1.2.10: Processing and consequences of mutant huntingtin. The presence of an expanded CAG repeat leads to protein misfolding. This could confer toxicity in several ways: 1) Misfolded huntingtin could lead to altered interactions with other proteins. 2) Mutant huntingtin forms aggregates within the cytoplasm, where it may recruit other proteins. 3) Misfolded huntingtin is susceptible to cleavage by caspases and calpains, leading to nuclear localisation and aggregation resulting in transcriptional dysregulation. 4) Degradation by the ubiquitin-proteasome system (UPS) becomes impaired due to clogging of the pore by the expanded glutamine repeats. (Adapted from Ciechanover & Brundin, 2003.)

CRE-regulated genes are known to be down-regulated early on in HD progress (discussed in Wyttenbach *et al.*, 2001). Likewise, CRE-mediated transcription is compromised by proteins that carry an expanded poly glutamine stretch (McCampbell *et al.*, 2000; Nucifora *et al.*, 2001; Wyttenbach *et al.*, 2001). The precise mechanism by which CRE-mediated gene transcription is impaired is not clear. CRE is activated by CRE binding protein (CBP), which has been identified within polyQ-containing protein aggregates (McCampbell *et al.*, 2000; Nucifora *et al.*, 2001). Mutant huntingtin has also been shown to interact with the glutamine-rich activation zone (Nucifora *et al.*, 2001) and the acetyltransferase domain (Steffan *et al.*, 2001) of CBP, thereby drawing CBP into aggregates and removing it from the system. Clearly loss of CBP could lead to decreased expression of CRE-mediated genes.

CRE-mediated transcription can also be activated by $TAF_{II}130$, which has also been shown at elevated levels within aggregates (Shimohata *et al.*, 2000). The precise role of $TAF_{II}130$ is not understood, but it has been argued that it may preferentially bind to expanded polyQ stretches thereby being recruited into aggregates (Shimohata *et al.*, 2000). Contrary to this notion it has been reported that $TAF_{II}130$ bind equally well to both wild-type and mutant huntingtin (Dunah *et al.*, 2002).

$TAF_{II}130$ is also implicated in the Sp1 transcription pathway. It appears that mutant huntingtin prevents the interaction of $TAF_{II}130$ with Sp1 and thereby impedes the interaction of Sp1 with DNA (Dunah *et al.*, 2002). Several gene products downstream of the Sp1 promoter are known to be reduced in HD. Particularly pertinent to neuronal dysfunction is the decrease in neurotransmitter receptors, such as D_2 dopamine receptors and a decrease in nerve growth factor (Cha

et al., 1998; Luthi-Carter *et al.*, 2000). Indeed, Sp1 has been shown to be reduced early in the human disease process (Dunah *et al.*, 2002; Li *et al.*, 2002) and before formation of visible aggregates in cell models (see Sugars & Rubinsztein, 2003). Importantly the expressions of some genes are unaltered. For example the axonal or dendritic neuronal markers microtubular associated protein 2 (MAP2) and neurofilaments, preprotachykinin (an mRNA expressed only the MSSNs) and the glial marker genes glial fibrillary acidic protein (GFAP), myelin basic protein and major histocompatability complex II (MHCII), all show normal mRNA levels. This suggests that the reduced mRNA levels observed is due to a specific, rather than generalised, impairment in transcription.

It appears, therefore, that abnormalities in transcriptional regulation may play an important role in the pattern of pathology seen in HD by down regulating protein expression. The impairment seen in transcription is not fully understood, but may be a result of activator impoundment into aggregates. Examples of genes that are relevant to neuronal activity and survival and show reductions in transcription (as detected by mRNA levels) in the R6/2 mouse model (see section 1.2.7) are listed in table 1.2.2. Two techniques have been used in the detection of mRNA levels: *in situ* hybridisation and DNA array. *In situ* hybridisation uses a labelled probe to detect mRNA within a section of tissue. The DNA array approach uses labelled complementary DNA prepared from mRNA extracted from dissected striata. cDNA abundance is then assessed of known DNA sequences.

mRNA	Striatum			Cortex			
	4 weeks	6 weeks	12 weeks	4 weeks	8 weeks	12 weeks	
mGluR1	77^{**}	49^{**}	51^{**}	91	81	73^{**}	*In situ* hybridisation
mGluR2	94		126	82	$56^{\S}$	$55^{\S}$	
mGluR3	100		69	84	77	$59^{\S\S}$	
mGluR5	93		84	113		115	
NMDA-NR1	87	72	105	93	90	97	
D_1 Dopamine R	$54^{\S}$	60^{**}	$38^{\S\S}$				
D_2 Dopamine rR	82	$39^{\dagger\S\S}$					
$PKC_{\beta II}$			$55^{\dagger\dagger **}$			$70^{\dagger *}$	
Enkephalin			16^{*}				
Substance P			N/C				
GAD (65/67)			N/C				
Protein Phosphatase Inhibitor 1		44■	39■				DNA array
Adenyl cyclase (V)		20■	15■				
Adenyl cyclase (II)		48■					
D_4 Dopamine R		24■					
PKC_{γ}		53■					
$PKC_{\beta II}$		77▫	53■				
GluR6		120▫	28■				
D_{2A} Dopamine R		63□	39□				
Protein phosphatase 1		50□					
Neuronal cannabinoid R (CB1)		67▫	40□				
α2 Adrenergic R		35▫	29□				
Ryanodine R		26■	20■				
K^+_{IR} channel		26■	29■				
$K^+_{\beta 1}$ channel subunit		59▫	56■				
VSCC		83▫	59□				
IP_3 R		46■	42■				
GAD		63▫	39□				
Preproenkephalin		56■	46■				
PP2B (calcineurin)		46■					
cAMP-dep PK inhibitor		39■					
Proteasome activator PA28		110▫	320□				
MHC I B(2)		120▫	270□				
MHC II		N/C▩	N/C▩				
MAP2		N/C▩	N/C▩				
Neurofilaments		N/C▩	N/C▩				
GFAP		N/C▩	N/C▩				

Table 1.2.2: mRNA levels in R6/2 mice. All values are expressed as a percentage of age-matched wild-type mice. *= P<0.05; **= P<0.01; §= P<0.001; §§= P<0.0001; †=8 week old mice; ††= 10 week old mice. ■= Decrease called in 4/4 comparisons; □= Decrease called in 3/4 comparisons; □= Increase called in 4/4 comparisons; ▫= Change in less than 3/4 comparisons; ▩= No change (N/C) observed. *In situ* hybridisation data adapted from Cha *et al.* (1998); Menalled *et al.* (2000); and Harris *et al.* (2001). Array data adapted from Luthi-Carter *et al.* (2000).

1.2.6.2 Transport

Protein expression at the nerve terminal is dependent on transport from the nucleus of either mRNA to the nerve terminal for *in situ* translation (Eberwine *et al.*, 2001; Steward & Worley, 2001) and/or transport of previously translated proteins and other molecules (Silverman *et al.*, 2001). The distance between the cell body (DNA source) and nerve terminal can often be a great distance and transport of gene products involves interaction with a number of molecules including the microtubule lattice, motor components and adaptor molecules (see figure 1.2.11).

Wild-type huntingtin has been implicated in vesicle transport along nerve processes. Huntingtin has been shown to purify with vesicle fractions from human and rat brain, to be associated with microtubules and is known to have interactions with proteins involved in transport (discussed in Jones, 2002).

The process of vesicle trafficking involves dynactin, which is an activator protein that is required by the motor protein dynein to move vesicles along microtubule networks. Dynein has adenosine triphosphate (ATP)ase activity which provides the energy required for the process. Dynactin is also associated with centractin, which in turn binds the vesicle for transportation (Allan, 1996, see figure 1.2.11). Huntingtin has also been shown to interact with the motor component of transport, dynactin, via a huntingtin interactor called huntingtin-associated protein 1 (HAP1, Engelender *et al.*, 1997; Li *et al.*, 1998, see figure 1.2.11). The interaction of huntingtin with these components suggests that huntingtin may play a role in neuronal transport. Transport may, therefore, be affected when mutant huntingtin is expressed. The effect of abnormal huntingtin on transport has been investigated by

expressing *N*-terminal fragments of human huntingtin carrying 2, 23, 62 or 100 Qs in the squid giant axon. Expanded, but not normal, polyQ repeats lead to inhibited axonal transport (Szebenyi *et al.*, 2003). Similar results have been obtained in the fruit fly *Drosophila melanogaster* (Gunawardena *et al.*, 2003).

The impairment in transport is through a direct decrease in the functions of huntingtin and its interacting proteins, rather than inclusion into aggregates. But clearly the observation that mutant huntingtin can indeed sequester wild-type huntingtin (and its interacting proteins) into aggregates could compound impairments in vesicle transport.

1.2.6.3 Endocytosis

Endocytosis is an important cellular process that occurs at several places within neurones. In particular, endocytosis is required at the synapse where it is involved in the removal of neurotransmitter from the synaptic cleft to enable further fast neurotransmission and also to replenish vesicle pools within the presynaptic bouton (see Jarousse & Kelly, 2001). Endocytosis also occurs at the transGolgi apparatus, where loaded vesicles fuse with other organelles. Retrograde transport of proteins from the nerve terminal to the soma also requires endocytosis where it may be involved in regulating neurotransmitter receptor expression.

There are clear interactions of huntingtin with endocytotic mechanisms. Huntingtin has been shown to interact with α-adaptins, endophilins and clathrin, all of which are involved in the endocytotic process, via HIP1 (Metzler *et al.*, 2001; Waelter *et al.*, 2001b).

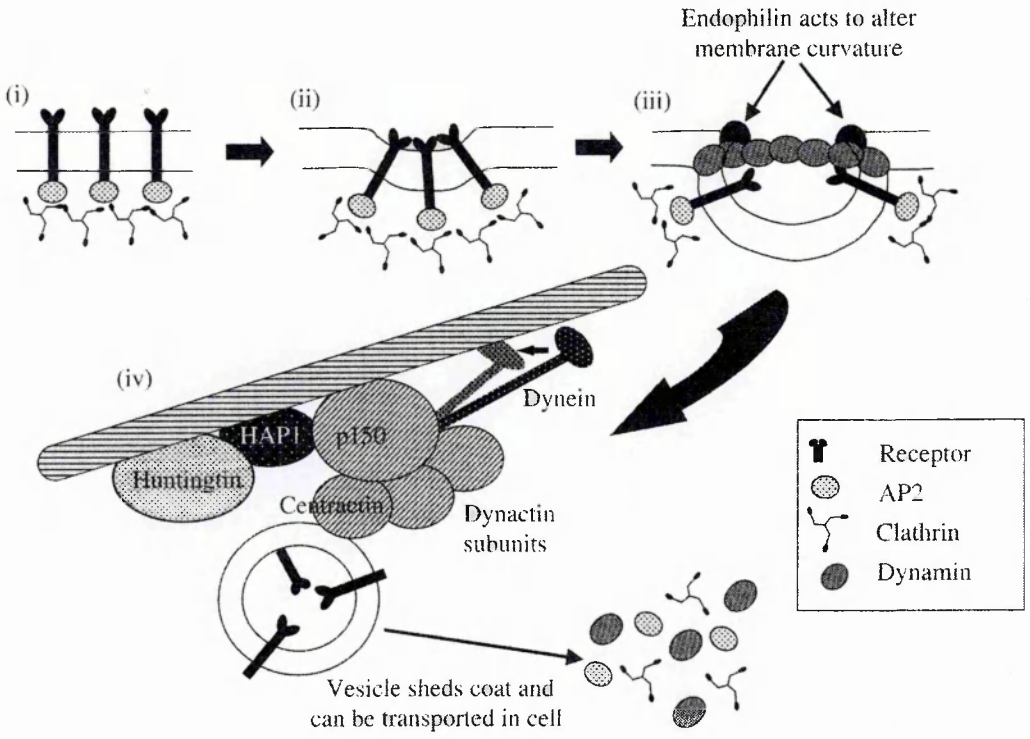


Figure 1.2.11: The role of huntingtin in vesicular transport along axonal processes. Following vesicle formation (i-iii), interaction with centractin binds the vesicle to a complex of proteins that mediate transport. One of these proteins is dynactin which interacts with dynein, an ATPase that provides energy for the process. Huntingtin is known to interact with dynactin though HAP1 and also interacts with microtubules, suggesting that huntingtin has a role in these processes. (From Jones, 2002.)

There are several examples of mutant huntingtin directly affecting endocytosis. Mutations of the yeast HIP1 homologue, Sla2p, disrupts endocytosis (Raths *et al.*, 1993); Knockout of the HIP1 gene in mice leads to decreased AMPA receptor trafficking though a decreased interaction with clathrin (Metzler *et al.*, 2003); *N*-terminal fragments of huntingtin have been shown to bind to synaptic vesicles preventing glutamate reuptake from the synaptic cleft (Li *et al.*, 2000a); also the interaction between huntingtin and post-synaptic density protein 95 is altered by the presence of expanded glutamine repeats. Post-synaptic density protein 95 is a scaffold protein that binds the NR2 subunit of the NMDA glutamate receptor in order to regulate cell-surface expression (Sun *et al.*, 2001).

1.2.6.4 Exocytosis

The release of intracellular chemicals may also be affected in HD. In particular, abnormalities in the proteins involved in exocytosis have been detected in a mouse model of HD expressing exon 1 of the HD gene (R6/2 mouse, see section 1.2.7)

Release of neurotransmitter from the presynaptic terminal is under the control of a number of proteins. The vesicle pool is bound to the cytoskeleton by synapsins that, once phosphorylated, allow mobilisation of the pool towards the membrane making them available for exocytosis. A number of proteins are involved in exocytotic membrane fusion, including synaptobrevin, syntaxin and SNAP-25, collectively known as SNARES. The cell membrane associated SNARES, SNAP-25 and syntaxin, fuse with the vesicle associated synaptobrevin to pull the two membranes together to cause exocytosis. The fusion of the SNARE complex is

mediated by the accessory proteins synaptotagmin, synaptophysin, rab3A, α-SNAP and complexins I and II (Mochida, 2000; Marz & Hanson, 2002). The complexin proteins are in direct competition with α-SNAP by providing an inhibitory role on neurotransmitter release (McMahon *et al.*, 1995).

Two of these accessory proteins are known to be affected in mouse models of HD. Complexin II expression levels are progressively reduced (Morton & Edwardson, 2001) and mutant huntingtin blocks exocytosis in PC12 cells by direct depletion of complexin II (Edwardson *et al.*, 2003). While expression levels of other SNARE and accessory proteins are normal (Bibb *et al.*, 2000; Morton & Edwardson, 2001), the phosphorylation state of synapsin 1 is increased (Liévens *et al.*, 2002).

Changes in the expression and phosphorylation states of proteins involved in exocytosis could underlie the decrease in glutamate release observed when mutant huntingtin is expressed in mice (Li *et al.*, 2003). The results of these experiments would predict a presynaptic alteration in synaptic transmission, and this will be discussed in section 1.4.

1.2.6.5 Loss versus gain of function

Genetic analysis suggests that a loss of function is not responsible for the HD phenotype. Knock-out of the gene (see Animal models section 1.2.7) does not result in an HD-like phenotype, suggesting they are caused by events other than loss of function. In the human condition, homozygosity does not appear to confer a more aggressive syndrome (Huntington's Disease Collaborative Research Group, 1993) again suggesting a gain of function.

Although the precise cellular function of huntingtin has yet to be elucidated, it appears (as discussed above) to interact with other cellular proteins, including huntingtin interacting proteins 1, 2 and 3 (HIP1; HIP2; HIP3), huntingtin associated protein (HAP1) and, amongst others, α-adaptin and endophilins. It has been suggested that the polyglutamine tract could confer a gain of function by altering the interaction of mutant huntingtin with its interacting proteins. Conversely there could also be a loss of function, whereby interactions are prevented by the misfolding and subsequent cleavage and aggregation of huntingtin and its associated proteins, including wild-type huntingtin. The loss of function could lead to cellular death by preventing the normal action of huntingtin in axonal transport and neurotransmitter release and thereby affecting synaptic dysfunction and excitotoxicity. It is likely, therefore, that the toxicity observed in Huntington's disease is a balance between both a gain and a loss of function (Prospero & Tagle, 2000).

1.2.7 Animal models of Huntington's disease

Several animal models of HD have been produced and, as with all animal models of human conditions, each has their relative merits and shortfalls. The first models were based on recreating the pattern of neuropathy seen at the end-stages of the human condition by targeting lesions in rats and primates. Following the identification of the gene responsible for HD (Huntington's Disease Collaborative Research Group, 1993), it was possible to target this gene to create genetically modified animal models of HD. The first were mice with the mouse *huntingtin* gene homologue (*Hdh*) knocked out. More sophisticated transgenic models have since

been produced, including invertebrates with a polyQ insertion, mice with either partial or full-length transgenes and also knock-in mice, where a gene insert is targeted to replace the mouse homologue gene *Hdh,* which shares 91% homology to human huntingtin (Barnes *et al.*, 1994; Lin *et al.*, 1994). More recently a rat model of HD has been generated. These models will be discussed in the following section, with a particular focus on the R6 line, which has the largest body of research associated and has been utilised in the present thesis.

1.2.7.1 Excitotoxic models

It is widely held that glutamate toxicity may underlie the loss of neuronal cells observed in the brains of HD patients. Inappropriate activity of glutamatergic inputs could lead to increased levels of glutamate and hence greater glutamate receptor activation. The key glutamate receptor involved in excitotoxicity is the NMDA receptor, which, when activated under a depolarised membrane state, allows calcium influx (Collingridge *et al.*, 1983b). The amount of calcium entering the cells of HD patients may be further enhanced by inappropriate activity of the NMDA receptor (Chen *et al.*, 1999b; Levine *et al.*, 1999; Zeron *et al.*, 2002). Prolonged elevation of intracellular calcium levels leads to cell swelling and damage to the Golgi apparatus, mitochondria and clumping of chromatin (Garthwaite, 1994) and glutamate toxicity could, therefore, contribute to neuronal cell death. It should be noted, however, that other mechanisms, such as oxidative stress, impaired metabolism and cellular dysfunction have also been implicated in HD neurotoxicity.

Rodents

In support of the glutamate toxicity model, injections of glutamate analogues into the striatum of rats provided the first models of HD that closely replicated the neuropathological damage occurring in HD (shown in figure 1.2.12) and also produced an HD-like phenotype including both motor and behavioural deficits (for example see Scattoni *et al.*, 2004). The first models used kainic acid (KA) injections which resulted in a similar loss of neurones to that observed in the HD condition. Projection neurones were lost, while interneurones were relatively spared and decreases in both the GABA synthetic enzyme glutamic acid decardoxylase and choline acetyltransferase were observed (Coyle & Schwarcz, 1976; McGeer & McGeer, 1976). Similar results were obtained with the glutamate analogue ibotenic acid (Isacson *et al.*, 1985).

A closer replication of the pathology observed in HD was created by injection of the NMDA receptor specific agonists quinolinic acid (Schwarcz *et al.*, 1983) or L-homocysteic acid (Beal *et al.*, 1990). Both quinolinic acid and L-homocysteic acid lesions in the rat striatum resulted in loss of spiny GABA and substance P neurones, but sparing of NADPH-diaphorase/nNOS/somatostatin/neuropeptide-Y-positive neurones.

Similarly to glutamate agonists, inhibitors of mitochondrial activity have been found to reproduce aspects of HD. Either systemic or local injection of aminooxyacetic acid, 3-nitropropionic acid (3-NP), malonate or azide have been shown to cause secondary excitotoxic lesions by inhibition of the mitochondrial respiratory chain (see Yohrling IV & Cha, 2002 for review). Malonate and 3-NP are both inhibitors of succinate dehydrogenase, which is complex II of the mitochondrial

electron transport chain. This disruption results in decreased ATP levels by preventing oxidative phosphorylation. Reduced ATP levels leads to cell depolarisation by effecting Na^+/K^+/ATPase activity. Cell depolarisation relieves the magnesium block on the NMDA receptor, again exposing the cell to excitotoxicity. Therefore the mechanism by which systemic administration of 3-NP gives rise to neuronal loss similar to that in HD is (possibly) two-fold: (1) altered energy metabolism may play a direct role; (2) reduced energy metabolism may compound the effects of excitotoxicity. How this mechanism confers selective neurotoxicity is unclear.

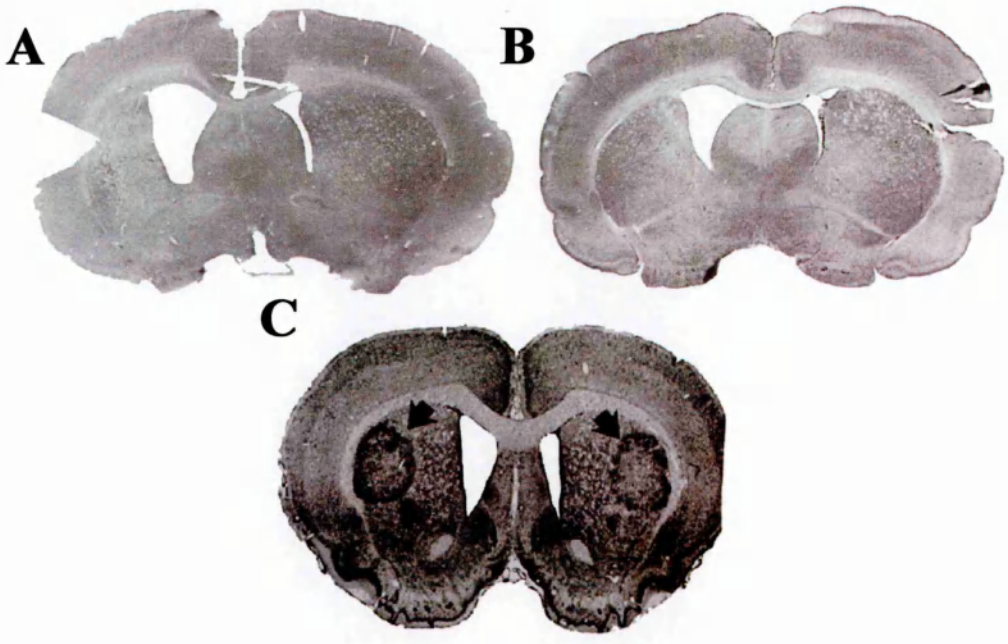


Figure 1.2.12: Excitotoxic lesions in rats. *A*: Focal lesion of the basal ganglia induced by injection of KA into the left hemisphere. *B*: Focal lesion induced by injection of quinolinic acid into the left hemisphere. Note the marked striatal atrophy on the injected (left) side compared to the contralateral hemisphere. *C*: Systemic administration of 3-NP leads to striatal lesions (arrows) and relative sparing of substance P-containing neurones. (A & B adapted from Beal *et al.*, 1991; C adapted from Reynolds *et al.*, 1998)

Primates

As the monkey striatum is more similar to the human striatum than the rat, neurotoxic models of HD have been expanded to include non-human primates. Models of chorea have been created by the intrastriatal injection of KA followed by the dopamine precursor L-DOPA (for example see Kanazawa *et al.*, 1986; Kanazawa *et al.*, 1993). Another primate model has been established by the injection of ibotenic acid into the baboon striatum (Hantraye *et al.*, 1992a). These models closely recapitulate the HD phenotype seen in humans. Injection of the mitochondrial inhibitors 3-NP or malonate into primates both caused selective lesioning of the caudate-putamen, choreic-like movements and, importantly, also impaired cognitive processes (for example see Schulz *et al.*, 1997).

While the excitotoxic lesions in rats and primates model the end stages of HD, they do not reproduce the progressive nature of the disorder and therefore early neuronal dysfunction is overlooked in these models. The identification of the gene responsible for HD has enabled the creation of superior models in the form of transgenic animals which have a developmental neurological phenotype.

1.2.7.2 Transgenic models

Recent developments have allowed the manipulation of the mouse embryo such that specific genetic mutations can be expressed. These include pronuclear injection, which involves microinjection of purified foreign DNA sequences into the nuclei of

preimplanted embryonic cells. The integration of the transgene occurs at a random site within the mouse genome and often multiple copies are incorporated at the same chromosomal locus. The zygote can then be implanted into pseudopregnant mothers, allowing the gene to be transmitted to offspring in a Mendelian fashion to future generations (see Brusa, 1999 for review).

A second way to produce genetically modified mice is by gene targeting by homologous recombination. Mutations are generated *in vitro* within undifferentiated embryonic stem cells. The rare homologous recombinant events are then selected for against the relatively high background of non-homologous recombinant events, usually using a positive-negative strategy. Targeted stem cells can then be injected into a host blastocyst and implanted into a pseudopregnant mother (again, for review see Brusa, 1999). This technology has enabled the development of reverse genetics: gene knock-out, where one gene is completely inactivated; or gene knock-in, where a targeted gene is substituted by a mutated form. Reverse genetics has enabled the investigation of gene function *in vivo*.

One problem in generating a progressive disease phenotype within a mouse model is time span. A laboratory mouse lives on average for only two years, while disease onset with a typical CAG expansion of 40 repeats is around 40 years. The juvenile form of HD, which is caused by exceptionally long CAG repeats, provides the opportunity to produce models of HD that potentially express a phenotype within the lifespan of the mouse.

Single cell models

The simplest of genetic model for Huntington's disease has been produced using single cells. A whole host of such models have been produced and these have/will be discussed with their appropriate findings. Briefly, immortal cell lines are used for their relative inertness, for example human embryonic kidney cells or PC12 cells. Inserts have included exon 1 fragments of human DNA, full length constructs of huntingtin and simple polyglutamine stretches. Repeats ranging from innocuous 15 CAGs through to 150 have been used. Most commonly the cytomegalovirus (CMV) is used as the promoter to drive expression (for review see Jones, 2002).

Mutant huntingtin has also been expressed in single cell organisms. For example full-length huntingtin with 25, 47, 72 or 103 glutamine residues has been expressed in yeast (Krobitsch & Lindquist, 2000). Huntingtin was co-expressed with green fluorescent protein which was used to visualise aggregate formation. It was found that polyQ repeat length highly correlated with aggregate formation such that the 103Q protein formed single large aggregates; the 25Q protein formed no aggregates; and the 72Q protein formed some aggregates while some protein remained non-aggregated (Krobitsch & Lindquist, 2000).

Flies and worms!

Several transgenic invertebrate models have been produced that contain ployQ tracts (see Bates & Benn, 2002 for review). Insertion into the fruit fly *Drosophila melanogaster* has been the most successful, with constructs including *N*-terminal fragments of huntingtin (Jackson *et al.*, 1998; Steffan *et al.*, 2001) and simple polyQ

tracts (Marsh *et al.*, 2000) usually targeted to the eye (see figure 1.2.13). These models have a progressive phenotype, demonstrating aggregate formation, neurodegeneration, a behavioural phonotype and lethality. This model has proved useful in identifying genes that modify the phenotype induced by the polyQ expansion and for a high-turn-over therapeutic screen.

The soil nematode *Caenorhabditis elegans* has been put to similar use to the *Drosophila*. *N*-terminal fragments of huntingtin with an expanded CAG repeat (Faber *et al.*, 1999; Parker *et al.*, 2001) or naked expanded polyQ repeats (two lines under the control of different promoters, Satyal *et al.*, 2000) have been inserted into the *C. elegans* genome to produce worms with a modified phenotype. All four of these worms have shown cytoplasmic aggregates, an example of which can be seen in figure 1.2.13.

Due to their high turn over, both invertebrate models lend themselves particularly well to screens for potential interacting genes and drug therapies. Indeed, identified drugs are now entering phase III clinical trials (see Bates & Hockly, 2003). It should be remembered, however, that these models may have significantly different pathways involved in the regulation of and that may be perturbed by the HD mutation in man (Rubinsztein, 2002).

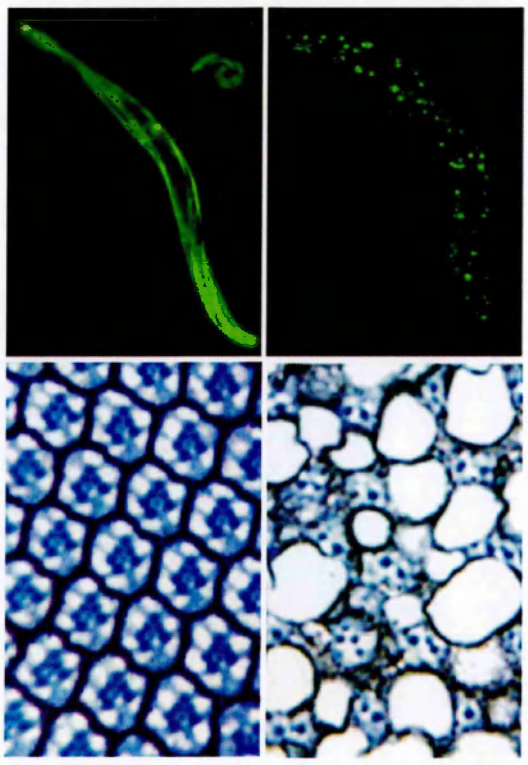


Figure 1.2.13: Invertebrate models of HD. *Top: C. elegans* expressing green fluorescent protein-tagged Q19 *(left)* and Q82 *(right)* which is expressed in the body wall muscle cells. Expanded polyglutamine tracts result in the formation of aggregates (Adapted from Satyal *et al.*, 2000.). *Bottom: D. melanogaster* ommatidia showing normal (wild-type) trapezoid arrangement with seven rhabdomeres *(left).* Expression of an expanded polyglutamine tract (Q108, *right)* results in large holes within the eye and only two rhabdomeres. (Adapted from Marsh *et al.*, 2000.)

Knock-out mice

Although these mice do not model the disease, knock-out of the mouse HD gene homologue *Hdh* has provided insight into the crucial developmental role of huntingtin. Knock-out mice have been generated independently by three separate groups (Duyao *et al.*, 1995; Nasir *et al.*, 1995; Zeitlin *et al.*, 1995). In all three cases nullizygous (–/–) mouse embryos died at approximately embryonic day E7.5. In contrast, heterozyotes (*Hdh*/–) were indistinguishable from littermate controls (Duyao *et al.*, 1995; Zeitlin *et al.*, 1995), indicating that HD is not caused by a haploinsuffiency (Bates & Murphy, 2002). The fact that complete inactivation of the gene results in a non HD-like phenotype favours a gain of function model for the human condition. This theory is supported further by the fact that mutant huntingtin can rescue the lethality wrought by gene-knockout (White *et al.*, 1997).

The severity of the knock-out phenotype means that this is the extent of the information gained from this model.

1.2.7.3 Truncated construct models

The transgenic models of HD can be separated into two groups: Those expressing full length constructs of the mutant HD gene; and those expressing truncated constructs. The R6 line was the first and most studied transgenic model produced and falls into the truncated category. I shall concentrate largely on the R6 lines, firstly because of the large body of evidence based on these models and secondly, because this being the mouse used in the current study.

R6 lines

R6 mice carry a fragment of human DNA containing exon 1 of the HD gene under the control of the human promoter sequence (Mangiarini *et al.*, 1996). The mice were created by microinjection of a 1.9 kb fragment from the 5' end of the human HD gene. The fragment was composed of approximately 1 kb of the 5' UTR promoter sequences, exon-1, including the CAG repeat and the first 262 bp of intron 1. Five lines showed ubiquitous tissue expression at less than endogenous levels and three of these lines had an expanded CAG repeat. The R6/1 (116 repeats), R6/2 (144 repeats) and R6/5 (128-156 repeats) lines each showed a progressive neurological phenotype, weight-loss and premature death, reminiscent of the human disorder. The other two lines that showed ubiquitous expression of huntingtin, Hdex6 and Hdex27, both have repeats within the normal human range and show no neurological phenotype.

R6/2

Of the lines that presented a neurological phenotype, the R6/2 line was considered the most interesting. This line most probably has three transgene integration sites, although two of these may have been silenced by deletions (Mangiarini *et al.*, 1996). There is a relatively high expression of the transgene at 75 *per cent* of the endogenous levels.

At weaning R6/2 mice are indistinguishable from their normal littermates. By the age of nine to eleven weeks, however, mutant mice start to display a progressively worsening phenotype (Mangiarini *et al.*, 1996). Dyskinesia (in the

form of limb clasping, see figure 1.2.14) in response to tail suspension is one of the first symptoms to manifest. Other components to the motor phenotype include a resting tremor that becomes worse under stress, choreic-like movements that may involve the whole body, stereotypic involuntary movements (e.g. repetitive stroking of the nose and face), mild ataxia and, as the phenotype progresses, epileptic seizure upon handling. The mutants are significantly underweight by 13 weeks (Carter *et al.*, 1999) and die of unknown causes by 16 weeks. At this age mutant mice can weigh as little as 60% of their littermate controls. Their brains are consistently ~30% smaller than controls. The key phenotypes are summarised in figure 1.4.2, where they are compared to the R6/1 mouse.

Huntingtin is expressed in all regions of the R6/2 brain. Expression is particularly rich in cortical regions with the highest levels seen in layer V pyramidal cells (Meade *et al.*, 2002). MSSNs of the striatum show moderate expression. Huntingtin has been shown to aggregate within the cytoplasm and nucleus of neuronal cells in the human condition (as discussed in section 1.2.4, DiFiglia *et al.*, 1997; Gutekunst *et al.*, 2002). Similarly, aggregates have been reported within the R6/2 mouse line (Davies *et al.*, 1997; Scherzinger *et al.*, 1997). Their emergence is progressive and they appear in a prescribed sequence, appearing in the cortex and hippocampus before the striatum (Morton *et al.*, 2000; Smith *et al.*, 2001). Within the cortical mantle, inclusions appear in a minority of cells by 3.5 weeks, with layers II/III and IV showing more inclusions than layer V. By four weeks there is a dramatic increase, with between 60 and 85% of cells containing aggregates. At nine weeks nearly all neurones have inclusion (Meade *et al.*, 2002). The progression of aggregate formation within the striatum follows a similar time scale (Meade *et al.*,

2002). Within the hippocampus inclusions appear in CA1 before CA3 and the dentate gyrus, a pattern that is maintained even in slice culture (Smith *et al.*, 2001).

A whole battery of tests, designed to assess the motor, behavioural and cognitive phenotype of the R6/2 line have been carried out. The mice show deficits in swimming and beam walking, poor performance on a rotarod, abnormal gait and reduced prepulse inhibition of an acoustic startle response (Carter *et al.*, 1999).

One demonstration that this mouse line is cognitively impaired came from a test of short-term memory, the T-maze. This test assesses alternation, based on the observation that rodents tend to spontaneously alternate pathways during exploration, maybe to optimise foraging (Dember & Fowler, 1958). The mice developed normally, showing no difference from littermates, however by five weeks, R6/2 mice made significantly fewer alternations than did their littermate controls. The mice could be encouraged to learn the process using visual cues (light and dark) and by nine weeks were exhibiting comparable alterations to that of littermates, indicating that the deficit was of short-term memory rather than visual deficits (Carter *et al.*, 1999). A spatial learning version of the T-maze was carried out by alternating the start arm. In this form of the task mice have to use spatial cues to learn the position of the reward. Control animals learn within 10 trials, while R6/2 mice only solve the task at low levels (Carter *et al.*, 1999).

Cognitive impairments within these mice have been confirmed in a test of spatial cognition, the Morris water maze (Morris, 1981). Initially mice at 7-8 weeks were tested and found to have slower escape latencies in a visual version of the test. In probe tests, the mutants spent significantly less time in the quadrant that previously contained the platform (Murphy *et al.*, 1998; 2000). It was possible that the apparent impairment in learning could be attributed the motor deficit rather than

an impairment in spatial learning *per se*. Nevertheless, the swim-path of the transgenics is clearly different than that of the littermate controls. As clearly shown in figure 1.2.14, the controls repeatedly return to the platform quadrant, while mutants swim in a more random path. The learning impairment was demonstrated to be real when similar experiments were repeated at an earlier stage and it was found that 3.5 week old mice also show an impairment in spatial learning, a time point before any motor deficits are apparent (Lione *et al.*, 1999).

Non-apoptotic neurodegeneration has been reported within the cingulate cortex of the R6/2 line (Turmaine *et al.*, 2000), although this is not detectable until long after an overt phenotype is established, suggesting that the behavioural phenotype is not a result of cell death, but rather neuronal dysfunction. As will be discussed in section 1.3, changes in synaptic plasticity are believed to underlie cognitive processes (Bliss & Collingridge, 1993). Indeed, impaired synaptic plasticity within the R6/2 line is widely reported (Levine *et al.*, 1996; Murphy *et al.*, 1998; 2000; Cepeda *et al.*, 2001; Klapstein *et al.*, 2001; Vetter *et al.*, 2003) suggesting that abnormalities in synaptic transmission and plasticity may underlie the changes in cognitive processing seen in humans with HD. Synaptic plasticity in the models of HD will be discussed in section 1.4.

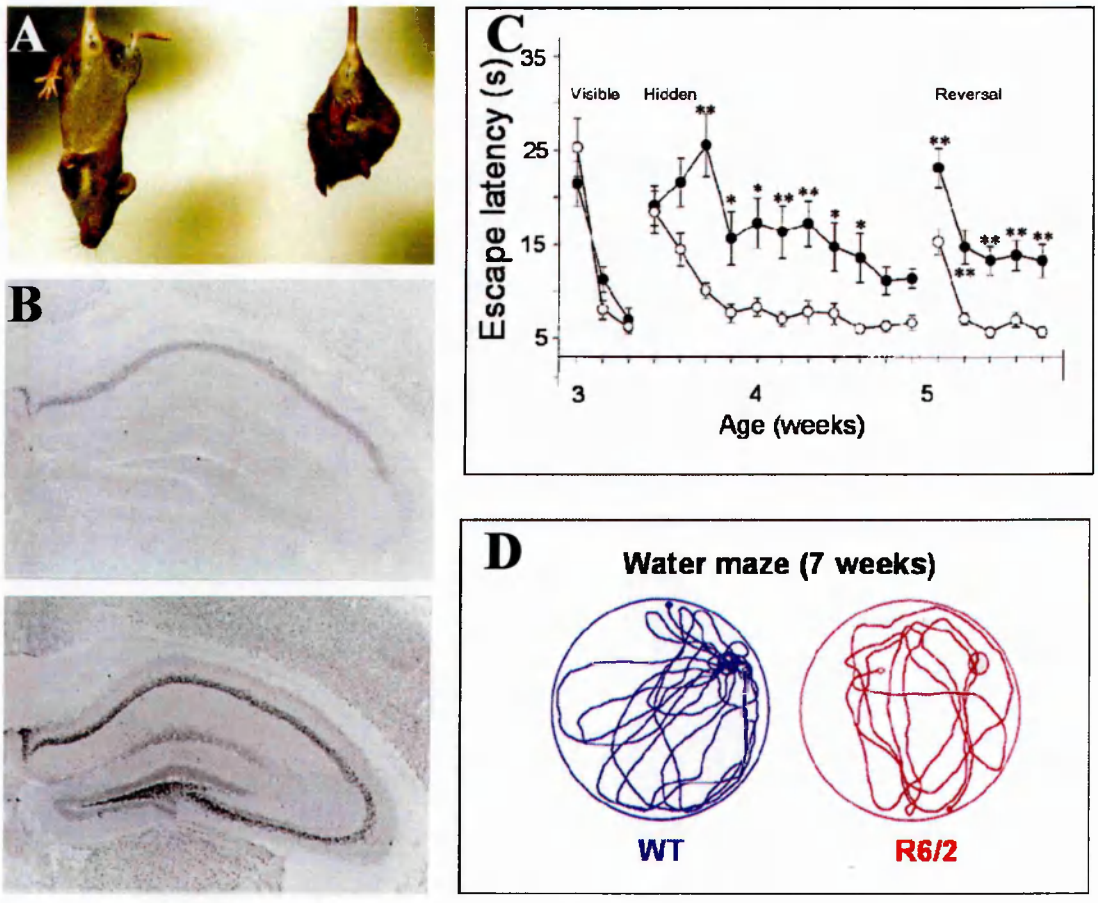


Figure 1.2.14: The R6/2 mouse. A, Hind-limb clasping. Wild-type littermates *(left)* show normal exploratory behaviour in response to tail suspension. In contrast, transgenic R6 mice show dyskinesia in the form of clasping. (Image from Mangiarini *et al.*, 1996.). B, Development of hippocampal and cortical aggregates at 3 weeks *(top)* and 7 weeks *(bottom)*. (Adapted from Morton *et al.*, 2000.) C, Cognitive impairments in the hippocampal-dependent test of spatial cognition, the Morris Water Maze. At three weeks of age both wild-type (○) and transgenic (●) mice show normal learning, expressed as a reduction in escape latencies for a visible platform. At 3.5 weeks, however, R6/2 mice show impairments in the hidden platform version of the water maze. Transgenics also show impairments upon reversal of the hidden platform quadrant (five weeks). (Adapted from Lione *et al.*, 1999.) D, Representative swim paths illustrating the impairment of spatial cognition during a probe test. Controls concentrate their search in the quadrant where the platform was placed during training, transgenic mice show a less focused swim path. (Adapted from Murphy *et al.*, 2000.)

R6/1

The R6/1 line was also interesting in that it showed a neurological phenotype similar to the human condition. Generally speaking, this strain is considered very similar to the R6/2 line, but importantly the phenotype is prolonged, with mice reaching end-stage at approximately fourteen months (see figure 1.4.2). The prolonged phenotype is likely to reflect the much lower expression levels of huntingtin in this line (31% of the endogenous levels, compared to 75% for the R6/2, Mangiarini *et al.*, 1996) more than the slightly lower CAG repeat length (116 and 156 in R6/1 and R6/2 respectively); although, given the strong correlation between these two parameters, there is likely to be some interaction.

R6/1 mutant mice begin to demonstrate the limb-clasping trait at about 14-20 weeks (Naver *et al.*, 2003). By the age of 22 weeks the transgenic mice have a significantly lower body weight than littermates. At this age R6/1 mice also show impairments in motor performance, particularly in an open-field arena, where they show less locomotion (Naver *et al.*, 2003; Bolivar *et al.*, 2004). They also show reduced levels of anxiety, as measured in the plus maze, where mutants show fewer entries into the arms overall and, moreover, less time in the closed arms (Naver *et al.*, 2003). R6/1 mice also show a higher rate of failures on the rotarod test, in an age dependent manner, compared to littermate controls (van Dellen *et al.*, 2000). Furthermore, the onset of this motor impairment can be delayed by environmental enrichment (van Dellen *et al.*, 2000; Glass *et al.*, 2004).

The only data available on cognitive testing within the R6/1 mouse model is within a thesis and some unpublished data that reports that barrel cortex-dependent discriminations, which require the mouse to distinguish two separate pathways using

only its vibrissae, is impaired in mice as young as 10 weeks old (Mazarakis, 2003; Cybulska-Klosowicz *et al.*, 2004, unpublished data) before the onset of a motor phenotype.

171 lines

Another mouse model of HD that utilises a truncated gene construct is the N171 model (Schilling *et al.*, 1999; Luthi-Carter *et al.*, 2000). This model expresses the first 171 amino acids of an *N*-terminally truncated huntingtin cDNA construct plus either 18 (N171-18Q), 44 (N171-44Q) or 82 (N171-82Q) glutamine residues. Expression is at lower than endogenous levels (10-20%) under the control of a mouse prion protein vector, which drives expression in almost all neurones within the CNS (Borchelt *et al.*, 1996). Similar to the R6 line, these mice develop a progressive neurological phenotype. At birth transgenics are indistinguishable from littermates, and develop apparently normally, but by two months the N171-82 mice fail to gain further weight. Mutant mice also start to show a resting tremor, hypokinesia, abnormal gait and hind limb clasping upon tail suspension and impairments on rotarod tests (Schilling *et al.*, 1999; 2001). Upon histological examination, neuronal intranuclear inclusions are clearly seen within the striatum, hippocampus and cortex of N171-82Q mice at end-stage (Schilling *et al.*, 1999). The life-span of the N171-82Q mice is reduced, ranging between 16 and 52 weeks, which probably reflects the level of expression in different founder lines (Schilling *et al.*, 1999).

HD lines

One more transgenic mouse model using a truncated gene insert has been made, known as the HD line (Laforet *et al.*, 2001). These mice express the *N*-terminal one third of the human HD gene with either normal (CT18) or expanded (HD46 and HD100) glutamine repeats. Expression of the transgene was under the control of the rat neurone specific enolase promoter, resulting in expression at less than that of native huntingtin. CT18 mice showed no neurological deficits, while from 3 months HD46 mice showed impairments in at least one of the tests used as assessment (clasping, rotarod, gait and activity); HD100 mice showed impairments in all of these tests (Laforet *et al.*, 2001). The phenotype is more benign than that of either the R6 or N171 models. Cortical and striatal neurones commonly had dysmorphic dendrites and showed cytoplasmic and nuclear accumulation of huntingtin. The degree of dysmorphic dendrites correlated well with the degree of neurological impairment.

Reversible model

An extremely interesting mouse model, in which the expression of mutant huntingtin can be reversed, has been produced. This conditional mouse model of HD was created by expressing exon 1 of the huntingtin gene under the control of a bidirectional tetO operator and the CMV promoter (Yamamoto *et al.*, 2000). The transgene contained a CAG repeat of 94 in one direction and a β-galactosidase (lacZ) reporter sequence in the other, thus allowing direct confirmation of the protein expression. The HD94 mice were then crossed with mice expressing the tetracycline

regulated transactivator under the control of the CaMKII promoter, which is necessary for the transcription of the tetO/CMV promoter.

Quantitative analysis of expression levels were not reported, although the protein is present within the striatum, hippocampus, cortex and, to a lesser extent, the amygdala and hypothalamus, but not within the cerebellum. Double transgenics displayed a clasping phenotype as early as four weeks and by 20 weeks a mild tremor was apparent. By 36 weeks these mice become hypokinetic. Double transgenics have a similar life span to wild types (two years). Nuclear aggregates become apparent by eight weeks. By 18 weeks brains of the double transgenic mice are consistently smaller than age-matched controls and there is distinct enlargement of the ventricles. Furthermore there was a decrease in D_1 dopamine receptor binding within the striatum (Yamamoto *et al.*, 2000).

At 18 weeks of age, half the mice were given doxycyclin, which binds to the tetracycline regulated transactivator and silences the gene expression ("gene-off"). Remarkably the gene-off group then go on to recover from the HD-like symptoms. They no longer display clasping and brain atrophy is halted. Inclusions within the cortex show a reduction and even completely disappear within the striatum. The reduction in D_1 dopamine receptor binding is also slightly reversed (Yamamoto *et al.*, 2000).

This model is important as it reveals that the progression of the disease is highly dependent on the *continuous* expression of the mutant protein and raises the hope that the disease may indeed be reversible, if not curable.

Rat model

More recently a rat model of HD carrying a truncated cDNA construct has been reported (von Hörsten *et al.*, 2003). The cDNA was formed from a 1962 bp fragment of HD gene obtained from a patient carrying 51 CAG repeats. The fragment is under the control of 855 bp of the endogenous rat promoter and is expressed at much lower levels than native huntingtin (values not available) in all tissues examined (von Hörsten *et al.*, 2003). Transgenic rats showed only a subtle motor phenotype: Dyskinetic and opisthotonos-like movements of the head. No tremor, ataxia, clasping or seizures were observed. They also show a lower gain in body weight than their littermates and, at 24 months, both males and females were 20% lighter. The transgenic rats started to die at around 15 months, although many survived well beyond the 24 months of the study.

While there were few motor abnormalities observed on home cage behaviour, specific behavioural tests did reveal deficits. At five months, rotarod activity revealed little difference from littermates. By 10 and 15 months, however, impairments in hind- and fore-limb coordination and balance were apparent. Anxiety levels were found to be reduced as early as two months, as shown by rats spending more time in the open arms of an elevated plus maze (von Hörsten *et al.*, 2003).

A clear cognitive decline was highlighted. Ten month old rats showed a poor performance on the spatial learning test of working memory, the radial maze (von Hörsten *et al.*, 2003).

Aggregate formation was apparent, particularly in the striatum, both within the neuropil and the nucleus. Aggregates were observed in 12, 18 and 24 month old rats, but not 1 or 6 month old.

One of the advantages of the rat model is its size. Being larger than mice, this model lends itself to magnetic resonance imaging, which has revealed progressive enlargements of the lateral ventricles and focal lesions of the striatum (von Hörsten *et al.*, 2003).

1.2.7.4 Full-length construct models

Two mouse models of HD have been produced that express the entire human huntingtin gene. Both mice demonstrate symptoms reminiscent of the human condition.

cDNA lines

The first full length model was created by microinjection of a 15 kb cDNA construct of the human gene that includes 10, 179 bp of HD cDNA that has been modified to include either 16, 48 or 89 CAG repeats (Reddy *et al.*, 1998; 1999). The promoter used to express the gene was the CMV in conjunction with an SV40 enhancer, resulting in high level expression at endogenous to five-fold endogenous levels. The protein was expressed in all tissues examined, including the brain, heart, spleen, kidney and gonads (Reddy *et al.*, 1998).

A progressive neurological phenotype developed in both the HD48 and HD89 lines. Similar to the truncated mouse models, the first phenotype to manifest was a limb clasp at eight weeks of age. The mice then progress through a hyperactive phase (characterised by unidirectional rotations, back flips and excessive grooming), followed by hypokinetic and then akinetic phases as the mice near to end-stage.

Degenerating neurones were observed in the striatum, cortex and hippocampus of both the mice carrying expanded repeats (Reddy *et al.*, 1998). Neurodegeneration was evident in mice in the hypokinetic and akinetic stages, but not before. Within the cortex, layer V pyramidal cells and, to a lesser extent, neurones of layers II, III and IV, undergo cell death (Reddy *et al.*, 1999) possibly though an apoptotic process (Reddy *et al.*, 1998). Inclusions were apparent in striatal, cortical, hippocampal and thalamic neurones of HD48 and HD89 mice from 12 weeks and throughout the hypo- and a-kinetic stages. Inclusions were also observed within the Purkinje cells of the cerebellum, but as in the human condition and other mouse models, Purkinje cells are relatively spared in this mouse.

The extremely high levels of expression calls into question the physiological relevance of this model as the true effects of the mutant gene may be masked.

YAC lines

A second full length model was achieved by the introduction of a human yeast artificial chromosome (YAC), containing 18, 46 or 72 (Hodgson *et al.*, 1999) and 128 (Slow *et al.*, 2003) glutamines into mice. The gene was under the control of its own promoter. Several lines were produced that carried 72 Qs. One mouse expressed the protein at three times the level of endogenous huntingtin (no line was

established). This mouse showed a neurological phenotype by 9 months, which included hyperkinesia (circling, similar to that seen in the cDNA lines), gait ataxia and foot clasping. When this mouse was sacrificed at 12 months there was a 50% reduction in body weight compared to wild-type controls and clear neurodegeneration and inclusions, particularly within the striatum, were detectable.

Another YAC72 mouse was able to be used as a founder to establish a breeding line. Mice from this line expressed protein at a much lower level (between one third and one half of endogenous levels). They showed no neurological phenotype and there was no evidence of aggregate formation. Despite the lack of an overt phenotype, there were electrophysiological abnormalities (Hodgson *et al.*, 1999) and these will be discussed in section 1.4.

The YAC128 line, produced in 2003 (Slow *et al.*), shows a similar behavioural phenotype to the more aggressive YAC72 line reported by the same group (Hodgson *et al.*, 1999), including a hyperactive phase followed by motor deficits and finally hypokinesia. The line that showed highest huntingtin protein expression (at 75% of the endogenous levels) was characterised further. Brain weight is significantly decreased compared to wild-type littermates at 9 and 12 months, but not at 6 months. More specifically striatal volume was decreased by 9 months and cortical volume by 12 months. Regarding aggregate formation, no clear inclusions were observed under light microscopy, however immuno-gold labelling for electron-microscopy did reveal small clusters of protein within the cytoplasm of striatal neurones from 12 month old mice. The motor deficit is correlated with the loss of striatal neurones, which show a trend towards loss at 9 months and are significantly reduced by 12 months.

1.2.7.5 Knock-in mice

The so called knock-in approach utilises gene-targeting to replace the sequence in exon 1 of the *Hdh* gene ($(CAG)_2CAA(CAG)_4$) with a CAG repeat within the pathological range in humans. Five such lines have been generated to date (see Bates & Murphy, 2002; Menalled & Chesselet, 2002; and Rubinsztein, 2002 for reviews). In two of the mice the sequence in the *Hdh* gene was directly replaced by a stretch of 72-80 (Shelbourne *et al.*, 1999) and 80 or 150 (Lin *et al.*, 2001) glutamine repeats. In the other three mice a hybrid gene, in which exon 1 of the mouse gene was replaced by exon 1 of the human gene carrying an expanded CAG repeat, was used. One of these mice has repeats of 20, 50, 92 and 111 Qs (Wheeler *et al.*, 1999), another has either 73 or 96 repeats (Levine *et al.*, 1999) and the last one has 79 glutamines (Ishiguro *et al.*, 2001). (It should be remembered that the number of glutamine repeats is two more than the number of CAG repeats due to the CAG stretch being followed by a CAA, CAG sequence (Huntington's Disease Collaborative Research Group, 1993); CAA also encodes glutamine.) By the nature of gene-targeting, the knock-in gene is under the control of the mouse promoter and therefore the gene is expressed in the context of the mouse gene.

Initially these mice appeared rather disappointing, with no overt neurological phenotype within the lifespan of the mice. With the exception of increased male aggression in the Shelbourne mouse (1999) (which is not likely to be a consequence of the mutant huntingtin gene, as this trait has not been reported in any of the other genetic mouse models of HD), the only neurological phenotype reported has been in the 150Q mouse which showed some limb clasping and gait abnormalities at 25 weeks of age (Lin *et al.*, 2001). Four of the five models show nuclear staining and

micro-aggregates (all except Levine *et al.*, 1999), while only one shows distinct inclusions (Lin *et al.*, 2001).

Despite the lack of an overt phenotype, there are molecular and cellular alterations within these mice. For example a decrease in the levels of mRNA for encephalin within the striatum of the 73Q and 96Q mice (Menalled *et al.*, 2000) and an increased sensitivity to NMDA within these same mice (Levine *et al.*, 1999) has been reported. Length-dependent CAG repeat instability has been reported in gametes of the Wheeler *et al* mouse (1999) and within somatic cells in the Ishiguro *et al* mouse (2001) including the liver, kidney, stomach and brain, but not cerebellum. The 111Q mouse reported by Wheeler *et al* has also shown to have an enhanced susceptibility to the mitochondrial complex II inhibitor 3-NP. Treating these mice with 3-NP leads to a greater degree of neuronal cell death within the striatum compared to wild-types (Ruan *et al.*, 2004).

The success of the transgenic and knock-in models of HD has meant that many new insights into the disease have been possible. Importantly, the generation of rodent models that exhibit a progressive phenotype similar to that of HD, including a cognitive impairment, has enabled electrophysiological studies, which would otherwise be impossible. Electrophysiological studies within these mouse models of HD will be discussed in section 1.4.

1.2.8 Summary

Huntington's disease is a devastating neurodegenerative disorder caused by an expansion in the CAG repeat in the gene encoding the protein huntingtin. HD has traditionally been considered a disease starting in midlife, characterised by motor and psychiatric symptoms and, on *post-mortem* examination, associated with marked neurodegenerative changes. It has now become apparent that prior to classical symptoms there are subtle cognitive deficits, particularly of recognition memory. Transgenic mouse models of HD have recently been generated. These mice show a phenotype similar to the human syndrome, including early cognitive deficits preceding an overt motor phenotype, suggesting that mouse models of HD may provide an appropriate experimental tool for examining changes in cognitive processing in presymptomatic HD. Learning and memory processes are thought to be encoded by plastic changes in the neural circuitry (Bliss & Collingridge, 1993). It is possible, therefore, that the early change in cognitive function in HD is attributable to alterations in synaptic plasticity. In order to explore changes in synaptic plasticity within these mouse models the next chapter will discus the mechanisms involved in normal synaptic plasticity.

1.3 Synaptic plasticity

The cellular processes involved in learning and memory are not fully understood, but it is widely held that long-term changes in the efficacy of synaptic-transmission within the central nervous system are involved. Both long-term increases and decreases in synaptic-transmission have been identified and are believed to have varying importance in different brain regions. Many of the mechanisms involved in inducing and expressing these plastic changes have been elucidated, particularly in the hippocampus, but also in other cortical and subcortical regions. The following chapter will explore some of the mechanism involved in these changes in order to understand how these may be altered in Huntington's disease.

1.3.1 A brief history of neuroscience

1.3.1.1 The brain and cognition

Today it is well established that the brain is the seat of cognitive function, however this was not always thought to be the case. Both the ancient Egyptians and Greeks held the notion that the primary organ of mental function was not the brain but rather the heart (Aristotle, c350 B.C.-a & c); the brain was thought to counterpoise the heart by cooling the blood (Aristotle, c350 B.C.-b). Hippocrates questioned this by stating that "Men ought to know that from nothing else but the brain comes... joys, delights, sorrows, griefs, despondency [and] in an especial manner, we acquire wisdom and knowledge..." (Hippocrates, c400 B.C.). During the Roman period it was proposed by the Greek physician Galen that the brain transforms sensory information or "vital

spirits" into *psychic pneuma*, or "animals spirits" and that these animal spirits are carried throughout the body by the nerves, which were thought to be hollow tubes (Galenus, c180 A.D.). Many of the ideas proposed by Galen endured well into the nineteenth century, when it was still believed that the nervous system was made up of a continuous network of nerves and that nerve growth was responsible for the formation and storage of memories. By the end of the century however, this view was challenged and it was suggested that the brain is in fact made up of separate elements, not in anatomical connection (Waldeyer-Hartz, 1891). Waldeyer termed these elements 'neurones' and thereby proposed the 'neurone theory'. Support for the neurone theory came soon after from the pioneering Spanish anatomist Santiago Ramón y Cajal, who, using the silver nitrate stain developed by Camillo Golgi (1873), demonstrated that the central nervous system is indeed made up of discontinuous elements- the neurones (Cajal, 1894). Furthermore, Cajal also showed that mature brain cells can no longer undergo cell division and rebirth, thereby eliminating this as the process by which learning and memory is encoded. (It should be noted that recent evidence shows that neurogenesis occurs throughout life, see van Praag *et al.*, 2002.) Based on these findings, Cajal suggested that the connections between the neurones may undergo modification and that these modifications may be part of the process of learning and memory. These neuronal connections were later characterised as specialised chemical junctions and were termed 'synapses' by the British physiologist, Sir Charles Sherrington (1897).

1.3.1.2 Hebb's postulate

The concept that learning could occur by changes at chemical synapses was popularised during the first part of the 20th century. In 1949 the Canadian Donald Hebb published a theoretical model of how the efficiency of transmission could be changed in an activity-dependent manner. In the virtual absence of any experimental evidence Hebb postulated that:

"*When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased*" (Hebb, 1949)

This statement could be reworded to say that the association of activity in pre- and post-synaptic neurones induces some change to bring about a strengthening in synaptic transmission. Evidence for this proposal was not found until the 1970s when an activity-dependent, long-lasting increase in synaptic efficacy was identified (see below). Increases in synaptic efficacy are now widely held to be the mechanism by which information is encoded within a neural network (Bliss & Collingridge, 1993).

1.3.1.3 Behavioural correlates of synaptic plasticity

Evidence that changes in synaptic transmission may be involved in learning and memory had previously been reported. The first of these studies was carried out by Sherrington (1898) who studied non-declarative (implicit) forms of learning. Non-declarative memories are those that do not require conscious recall. Sherrington showed that stimulation of the cats paw results in a reflex withdrawal of that limb. Following repetitive stimulation, however, the reflex withdrawal decreases, an observation called 'habituation'. Habituation has been shown to involve a reduction in the underlying postsynaptic potential (PSP) recorded in the motor neurone (Thompson & Spencer, 1966), suggesting that a reduction in synaptic transmission is responsible. This system was, however, too complex to dissect the precise synaptic mechanism.

In contrast to the cat, which contains several million neurones, the marine snail *Aplysia californica* has approximately 20, 000, making it a much simpler system. The *Aplysia* demonstrates habituation of the reflexive siphon withdrawal following repetitive stimulation. Habituation is homosynaptic and involves a decrease in the release of excitatory neurotransmitter, glutamate, from the presynaptic terminal, resulting in a smaller PSP in the motor neurones controlling the siphon (see Kandel & Schwartz, 1982; Abrams, 1994a, b).

Conversely, the *Aplysia* withdrawal reflex can be increased in another form of non-associative learning called 'sensitisation'. Following exposure to a single, but powerful noxious stimulus (for example a strong stimulus to the tail), subsequent stimulation of the siphon results in a heightened withdrawal. This is mediated by a

heterosynaptic mechanism that activates facilitatory interneurones that contain 5-hydroxytryptamine (5-HT; serotonin). The release of 5-HT activates G protein-coupled receptors located on presynaptic terminals of the sensory neurones. Activation of these receptors increases adenylyl cyclase activity and generates cyclic 3',5'-adenosine monophosphate. One target of cAMP is protein kinase A (PKA) which phosphorylates a variety of proteins, including potassium channels. Potassium channel phosphorylation causes them to close, thereby decreasing potassium ion efflux and thus broadens the action potential. A broader action potential results in greater calcium influx into the presynaptic terminal affecting greater neurotransmitter release (reviewed in Kandel & Schwartz, 1982; Abrams, 1994a, b).

The non-associative forms of learning are so called because they involve the activation of only one synaptic input at a time. In contrast, classical conditioning involves the simultaneous activity of two sets of synapses i.e. an associative form of learning. Associative learning is an example of "Hebbian" learning and the best known example of this was described by Ivan Pavlov (1927). Pavlov presented dogs with food (unconditioned stimulus; US), causing a salivary response. The presentation of a neutral stimulus (conditioned stimulus; CS), for example the sound of a metronome, did not cause salivation. Following training, consisting of repetitive pairing of the US and the CS, the sound of the metronome alone was able to elicit salivation. This shows that the dogs can learn to associate the metronome with the presentation of food. Importantly the interval between the CS and US was a critical factor such that the CS must precede the US by 300-500 ms otherwise pairing was ineffective.

Classical conditioning has also been demonstrated in the *Aplysia* and the mechanisms underlying this implicit learning has been identified. Associative

learning was achieved by pairing tail stimulation (US) with simultaneous stimulation of the siphon (CS), resulting in an enhanced withdrawal reflex. Repeated CS application resulted in increased calcium levels in the presynaptic terminal, leading to activation of a calcium-dependent adenylyl cyclase and thus enhanced production of cAMP produced by the US. In this way cAMP acts as a coincidence detector where the US and CS pathways converge (for review see Kandel & Schwartz, 1982; Abrams, 1994a, b).

Although the mechanisms involved in implicit learning in the relatively simple model of the *Aplysia* was readily identifiable, the ultimate goal was to identify synaptic mechanisms involved in the more complex mammalian brain. Mammals have explicit (declarative) forms of memories, which involve a conscious recall.

1.3.1.4 The hippocampus and long-term potentiation

The first direct evidence for Hebbian synapses in mammals came in the 1970s, when it was reported that activity dependent and enduring increases in synaptic efficacy could be induced in a region of the medial temporal lobe called the hippocampus (Lømo, 1966; Bliss & Lømo, 1973; Bliss & Gardner-Medwin, 1973). This experimentally-induced phenomenon of long-lasting synaptic enhancement has since been termed long-term potentiation (LTP). LTP and its counterpart long-term depression (LTD) are now the leading models for the synaptic and cellular processes underlying learning and memory (for review see Bliss & Collingridge, 1993; Bear, 1999).

Why the hippocampus?

Evidence from human studies has shown that the hippocampus is important in the processing of episodic memory (the memory of places and events). This role was realised when a patient, called H. M. for anonymity, had part of his temporal lobe, including the hippocampus, bilaterally removed as a treatment for intractable epilepsy. Following surgery, H. M. no longer suffered epilepsy, but was unable to recall events two years prior to and events following the surgery. H. M. was, however, able to recall distant events (Scolville & Milner, 1957). This type of memory loss is known as anteriograde amnesia and similar memory deficits can be induced in primates following hippocampal lesions (Mishkin, 1978).

Another benefit of using the hippocampus for the study of neural correlates of memory is its relatively simple, laminate structure (figure 1.3.1). The hippocampus comprises a trisynaptic circuitry, receiving inputs from the entorhinal cortex via the perforant pathway, which synapses onto granule cells of the dentate region. The mossy fibres of the granule cells project to the CA3 region where they form synapses with pyramidal cells. The efferents of these cells are known as the Schaffer collaterals and synapse with CA1 pyramidal cells. The simple circuitry and laminate structure means that the sinks and sources are known and that monosynaptic responses can be recorded in response to electrical stimulation.

The hippocampus, therefore, provides a system where memories are known to be processed and also possesses a simple architecture giving rise to easily interpretable synaptic currents.

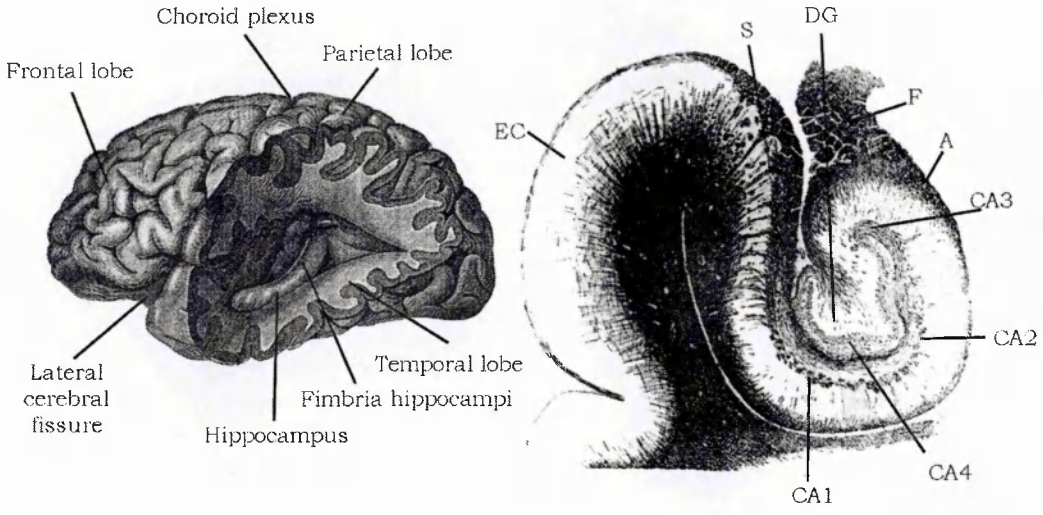


Figure 1.3.1: The human hippocampus. *Left,* Lateral view of the human cerebral mantle. The temporal lobe has been dissected to reveal the underlying hippocampus. (Adapted from Gray, 1918.) *Right,* Transverse section of the human hippocampus. The simple laminar structure of the hippocampus is clear, created by distinct cell body layers and their dendrites. A, alveus; CA1-4, fields of cornu ammonis; DG, dentate gyrus; EC, entorhinal cortex; F, fimbria; S, subiculum. (Adapted from Cajal, 1901.)

Long-term potentiation

Evidence for Hebb's postulate that a lasting enhancement in synaptic activity would occur following coincident activity at pre- and postsynaptic cells finally came in the early 1970s. Bliss and co-workers described a long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetised (Bliss & Lømo, 1973) and unanaesthetised (Bliss & Gardner-Medwin, 1973) rabbit following high-frequency stimulation of the perforant pathway. The conditioning paradigm used to induce LTP was based on the preliminary work on frequency potentiation by Terje Lømo that demonstrated an increased level of neuronal firing during and shortly after repetitive stimulation (Lømo, 1966). Such a conditioning stimulus is used to mimic neuronal activity during a learning event. A relatively low-frequency (10-15 Hz; cf. the 100 Hz often used today) conditioning stimulus, applied for several seconds, resulted in a series of events including post-tetanic potentiation and a period of depression which was followed by potentiation lasting up to ten hours in the anaesthetised animal and up to 16 weeks in the unanaesthetised animal. Furthermore a control pathway, which did not receive any conditioning stimuli, did not show potentiation. Here was a model that was not only long-lasting, but was also pathway specific, making it an attractive model for processes underlying memory. The phrase "Long-Term Potentiation" was coined in 1975 following a replication of the Bliss and Gardner-Medwin experiments within the rat by Douglas and Goddard (1975). To induce LTP they used a much higher frequency conditioning stimulus than that used by Bliss and colleagues. This high frequency stimulation (200-400 Hz) was thought to mimic the actions of central neurones more closely than the 10-15 Hz used in the earlier experiments and was found to more reliably induce LTP.

Brain slices

The initial studies on LTP were carried out using *in vivo* recording techniques. Although such recording may be related closely to events that occur within the brain, there are associated problems with this technique, in particular that of availability to laboratories. *In vivo* recording requires suitable equipment for monitoring animal health and having to maintain animals with implanted electrodes is costly and (arguably) stressful to the animal. Chronically implanted electrodes may also lead to gliosis around the site, calling into question the credibility of results. Similarly, movement of electrodes can occur over time, causing changes in responses.

A technique that made studying synaptic plasticity far more readily available to many laboratories was the *in vitro* slice. In the mid 1960s it was shown that slices of piriform cortex, taken from the guinea-pig, can be kept alive by submerging them in an oxygenated glucose-saline medium within a recording bath. The responses recorded were found to be similar to those obtained *in vivo* (Yamamoto & McIlwain, 1966). The *in vitro* slice technique has proved successful in other brain regions, including the hippocampus (Skrede & Westgaard, 1971), thus allowing this relatively simple structure to be studied without the need of maintaining animals with implanted electrodes. Furthermore, by using slices, the simplicity of the preparation can be kept to a minimum by isolating only the area of interest. A slice preparation also allows rapid application of pharmacological agents, whereas administration of these agents to intact animals can have added complications, such as metabolism effects or damage by canulation.

The first demonstration of LTP in the *in vitro* hippocampal slice came in 1975, showing a large potentiation of the pop-spike, recorded within CA1, following high-frequency stimulation (Schwartzkroin & Wester, 1975).

1.3.1.5 LTP: A neural correlate for memory?

Following the discovery of long-term potentiation several questions arose, the most pertinent, perhaps, being "Is it real?". Quite clearly this matter had to be addressed. In order for LTP (and long-term depression) to be fully established as a mechanism for learning and memory four criteria have been proposed (Martin & Morris, 2002):

(i) DETECTABILITY: Any changes in synaptic weights should be detectable following learning.

(ii) ANTEROGRADE ALTERATION: Altering the mechanisms of induction and/or expression of synaptic plasticity should also alter the ability to learn.

(iii) RETROGRADE ALTERATION: Altering the pattern of synaptic weighting following a learning experience ought to affect the ability for the animal to remember that previous learning event.

(iv) MIMICRY: A learning event could be mimicked by altering the appropriate synaptic weights, making the animal behave as though it had learnt.

Three of these criteria have, at least partially, been met, while the last (and perhaps the most fanciful of them), the ability to create a memory, has yet to be achieved. For a full review of the evidence for each of the criteria see the reviews by Martin and Morris (2000; 2002; 2003).

Many studies have been carried out to identify synaptic changes following learning. The clearest evidence for an LTP-like phenomenon following learning is within the primary motor cortex. Rats were trained to retrieve food from a small hole in a plastic box using their preferred paw. Following several days training motor cortical slices were prepared from both ipsilateral and contralateral hemispheres. Synaptic responses recorded from the contralateral side were consistently larger than those from the ipsilateral side in slices from trained rats. No differences were detectable between hemispheres in slices obtained from untrained animals (Rioult-Pedotti *et al.*, 1998). Furthermore, the capacity for LTP induction was reduced in rats that had undergone motor training (Rioult-Pedotti *et al.*, 2000). Similarly hippocampal-dependent learning tasks have been shown to increase EPSP size (Sacchetti *et al.*, 2001) and occluded further induction of LTP (Sacchetti *et al.*, 2002). These data suggest that LTP and learning may share common mechanisms.

Evidence that interference of the mechanisms of LTP induction affects learning and memory (anterograde alteration) largely arises from tests to assess spatial memory. The first was work within the watermaze developed by Richard Morris in the early 1980s (Morris, 1981; 1982). The watermaze is a learning task where a rat or mouse learns the location of a hidden platform within a pool of water. Once the platform has been removed, the animal's ability to remember the platform location is assessed by its swim path. The ability to succeed in this learning task is dependent upon the hippocampus, as demonstrated by the fact that lesions of this brain region severely impair an animal's ability to perform within the watermaze (Morris *et al.*, 1982). The NMDA receptor antagonist, AP5, previously demonstrated to block hippocampal LTP induction (Collingridge *et al.*, 1983b, see section 1.3.2), also impairs hippocampal-dependent place navigation in the water-

maze (Morris *et al.*, 1986). Secondly, prior electrophysiological saturation of LTP impairs subsequent, but not established, spatial learning (McNaughton *et al.*, 1986). Since these initial experiments, several independent research groups have demonstrated similar findings (e.g. see Moser *et al.*, 1998), supporting the idea that interfering with the induction of synaptic plasticity does indeed impair learning.

McNaughton (1986) also demonstrated that retrograde disruption, by high-frequency stimulation, was possible for recently learnt locations, but not established ones. Similar reports have since confirmed these findings and shown also that rats can subsequently learn new locations (Otnæss *et al.*, 1999; Brun *et al.*, 2001).

Attempts to mimic learning by inducing LTP have yet to prove successful. One approach was to try to mimic the activity within the neuronal circuitry and thereby replicate learning. A pattern of electrical stimuli, applied to the lateral olfactory tract, was used in an attempt to recreate odour sniffing in rats and thereby make rats "think" they had learnt (Roman *et al.*, 1987). Indeed, it was found that stimulation of the lateral olfactory tract can be substituted for real odours in an olfactory discrimination task. Long-term potentiation was not, however, detected in these rats (Roman *et al.*, 1987), but it was realised that the pattern of stimulation used in attempting to create the memories closely resembled the natural rhythmic activity of neuronal outputs seen within the hippocampus, known as theta rhythms (see Lynch, 2003). The pattern of stimulation was therefore renamed Theta Burst Stimulation (TBS) and has been shown to induce a robust form of LTP (Larson & Lynch, 1986).

In light of these results LTP appears as a very attractive candidate as the cellular mechanism underlying learning and memory processes.

1.3.1.6 Properties of LTP

By definition, LTP is long-lasting. The initial reports by Bliss *et al.* demonstrated LTP lasting for hours in the anaesthetised animal (Bliss & Lømo, 1973) and up to 16 weeks in the unanaesthetised animal (Bliss & Gardner-Medwin, 1973). Since these initial reports LTP has been shown to be extremely stable and has been found to last for over one year in the rat dentate gyrus (Abraham *et al.*, 2002).

LTP is often quoted as having three basic properties: Cooperativity, associativity and input specificity (Bliss & Collingridge, 1993). Somewhat confusingly, however, both cooperativity and associativity describe the same phenomenon; the two names have arisen from groups presenting the same findings, achieved using different stimulation paradigms (see McNaughton, 2003). Associativity/cooperativity describes how a weak input, at a subthreshold level for LTP induction, can be potentiated if it is active at the same time as other inputs, thus bringing that input above threshold (McNaughton *et al.*, 1978; Levy & Steward, 1979). LTP is also considered input-specific, in that synapses that are not active at the time of induction are (generally) not potentiated (Bliss & Lømo, 1973; Andersen *et al.*, 1977; Lynch *et al.*, 1977; but see Bradler & Barrionuevo, 1989).

Another characteristic of LTP is its reversibility. Previously potentiated synaptic responses can consequently be reduced by the application of a low-frequency conditioning paradigm, a phenomenon known as depotentiation (Barrionuevo *et al.*, 1980; Staubli & Lynch, 1990; Fujii *et al.*, 1991; Milner *et al.*, 2004b). This has been suggested as being a mechanism by which synapses can be "reset" (Dudek & Bear, 1993) thereby preventing saturation. The properties of LTP are shown in figure 1.3.2.

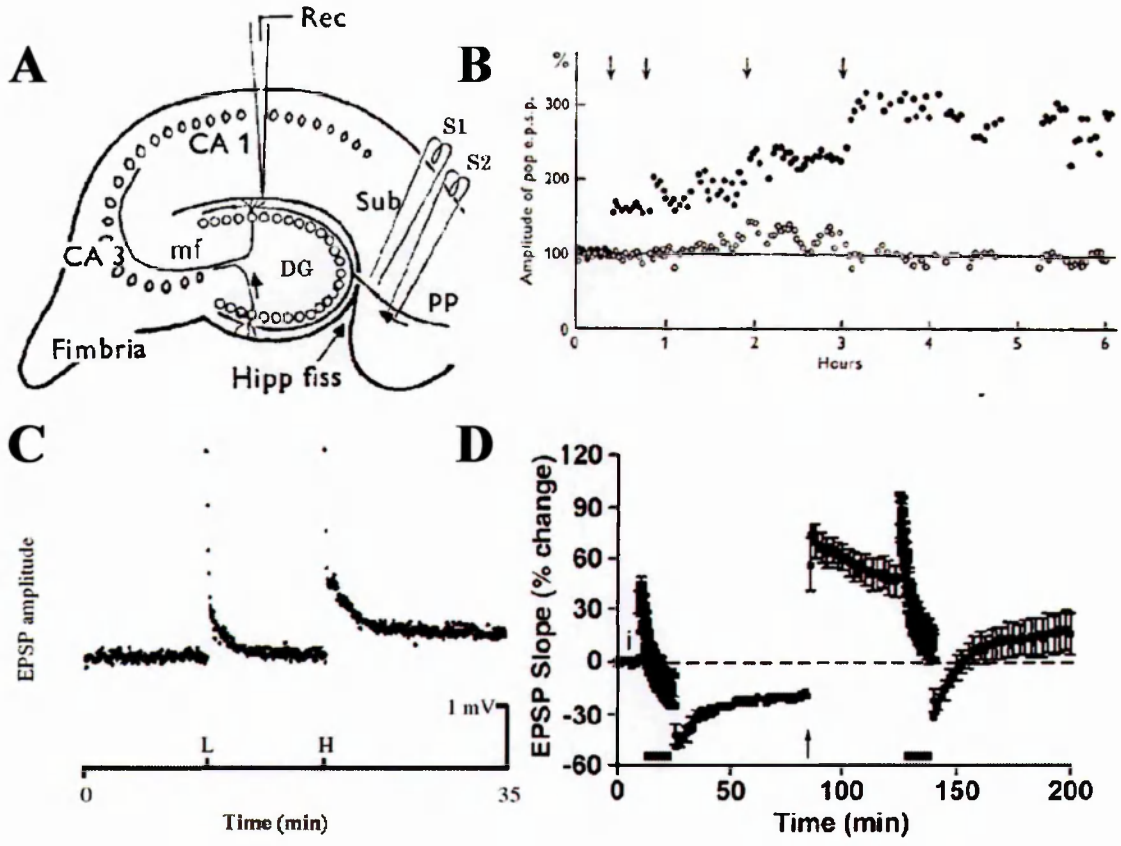


Figure 1.3.2: The properties of LTP. *A.* Simplified diagram of the rodent hippocampus. Test-shocks applied to independent perforant pathway (PP) inputs (S1 and S2) gives rise to EPSPs. DG, dentate gyrus; mf, mossy fibres; Sub, subiculum. *B.* The first demonstration of LTP as shown in the anesthetised rabbit dentate area. Following tetanus (arrows) to the S1 pathway (•), that pathway demonstrates LTP, while the control pathway (S2; ○), which receives no conditioning, remains at baseline, demonstrating input-specificity. (A & B adapted from Bliss & Lømo, 1973.) *C.* Cooperativity. A weak tetanus (L) applied to S1 induces a large increase in EPSP amplitude, but is only transient. Co-application of a strong tetanus (H) to S2 at the same time as a weak tetanus to S1 induces a long-lasting potentiation in the S1 pathway (Adapted from McNaughton, 2003.) *D.* Bi-directional plasticity. Application of a low-frequency conditioning paradigm (solid bar) leads to the induction of long-term depression (see section 1.3.3). Application of a high-frequency conditioning paradigm subsequently induces LTP. LTP can then be reversed using further low-frequency stimulation, resulting in depotentiation. (Adapted from Milner *et al.*, 2004b)

1.3.2.7 Short-term plasticity

While long-term synaptic plasticity is likely to underlie cognitive functions and memory storage, there are also short-term forms of synaptic plasticity. One example of this is facilitation, which describes how, following a single synaptic event, excitatory synapses remain in a state that allows more neurotransmitter release. However, this only lasts for a short period, of around a hundred milliseconds (Feng, 1941). Application of a short burst of stimulation leads to a marked increase in synaptic transmitter release that lasts up to a few seconds following the tetanus. This was first reported by Cragg & Hamlyn (1955) and later termed augmentation by Magleby (Magleby & Zengel, 1976). Post-tetanic potentiation (PTP) describes the increase in neurotransmitter release that lasts for minutes following a period of high-frequency synaptic activity (Feng, 1941) and, prior to the identification of LTP, was the main player for the mechanism underlying learning in such areas as the spinal cord (Eccles, 1953).

While these mechanisms may be involved in physiological functions at the synapse, they are far too transitory a mechanism for the long-term storage of information.

1.3.2 Mechanisms of LTP induction

LTP is usually induced experimentally by applying a high frequency stimulation paradigm to the afferent neurones. Three paradigms are commonly used. Tetanus (e.g. 100 stimuli at 100 Hz), theta-burst stimulation (e.g. several bursts of four

stimuli at 100 Hz delivered at an inter-burst interval of 200 ms) and primed-burst stimulation (e.g. a single priming stimulus followed at 200 ms by a single burst of four shocks at 100 Hz) (Bliss & Collingridge, 1993). The significance of these paradigms is that they mimic learning-related neuronal firing patterns seen within the hippocampus during learning (Otto *et al.*, 1991).

1.3.2.1 The glutamatergic synapse

In order to discuss the mechanisms involved in the induction of LTP, the structure and function of the central synapse under basal transmission needs to be considered. L-glutamate is known to be the most common excitatory neurotransmitter within the central nervous system (Collingridge *et al.*, 1983b). Glutamate, released from the presynaptic terminal, diffuses across the synaptic cleft where it activates a range of glutamate receptors. The main post-synaptic receptor activated is a ligand gated ion channel, known as the α-amino-3-hydroxy-5-methyl-4-isoazolepropionate (AMPA) class of glutamate receptors. There are four mRNA transcripts (GluR1-4) that give rise to AMPA receptor subunits (Gasic & Hollmann, 1992). Splice variants of these transcripts then form tetrameric transmembrane proteins with an intracellular carboxy terminal and an extracellular amino terminal. AMPA receptors are of the ionotropic type of receptor and are predominantly permeable to sodium (influx) and potassium (efflux). AMPA receptors that lack the GluR2 subunit are also, to a degree, permeable to calcium ions (Gasic & Hollmann, 1992). Kainate receptors are also present at the synapse and, like AMPA receptors, are principally permeable to sodium ions. KA receptors are comprised of the subunits GluR5-7 and KA-1 and 2

(Chittajallu *et al.*, 1999). AMPA/KA receptors are the main mediators of the postsynaptic response, known as an excitatory postsynaptic potential (EPSP). Blockade of these receptors by the AMPA/KA receptor antagonist 6-cyano-7-nitroquinoxaline-2, 3-dione disodium (CNQX) abolishes the postsynaptic response. There is also another class of ionotropic glutamate receptor, the *N*-methyl-D-aspartate (NMDA) receptor (Collingridge *et al.*, 1983b) which allows sodium and potassium flux, but is also highly permeable to calcium ions. The NMDA receptor does not play a role in basal synaptic transmission, but comes into play at high levels of activity. NMDA receptors are thought to be either a tetramer or pentamer comprising of assemblies of the NR1 subunit and a combination of NR2 subunits, which can be the product of four separate genes, NR2A-D. In addition to glutamate, the NMDA receptor requires the co-agonist glycine to function. The glycine site also binds polyamines that can modulate the function of the channel. Importantly, at resting membrane potential, the ion pore is blocked by a magnesium ion, which is expelled upon depolarisation. The role of the NMDA receptor in LTP induction will be discussed in section 1.3.2.3. One more class of glutamate receptor also exists. This last group is the G-protein coupled (metabotropic) class of glutamate (mGlu) receptors, of which there are eight known receptor clones, which fall into three broad classes. The role of mGlu receptors in synaptic plasticity is discussed in section 1.3.2.6.

The modern day view of a glutamatergic synapse within the CNS is shown in figure 1.3.3.

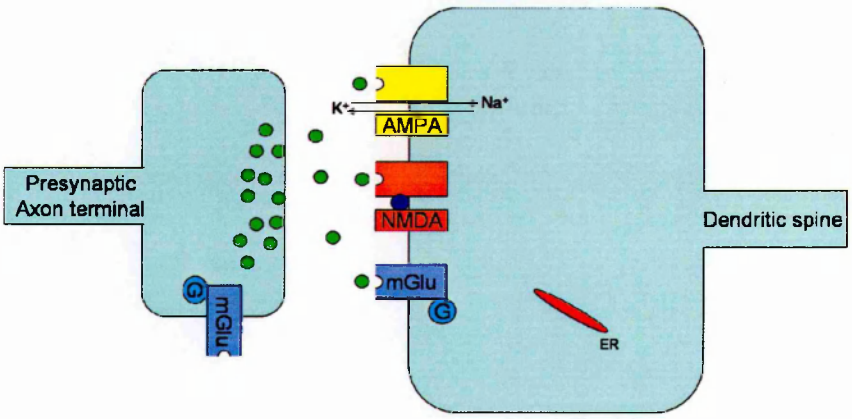


Figure 1.3.3: The glutamatergic synapse. L-glutamate (•), released from the presynaptic nerve terminal, diffuses across the synaptic cleft and binds with glutamate receptors. Activation of the AMPA/KA subclass of receptor leads to sodium influx and potassium efflux. Under basal conditions, opening of the NMDA channel by glutamate is prevented by a magnesium ion (•) within the pore. Activation of presynaptic metabotropic glutamate receptors may regulate the release of further transmitter. Relative proportions of receptors are not indicated.

1.3.2.2 Calcium influx

The high-frequency conditioning paradigms lead to cellular events that bring about the induction of LTP. The expression of LTP is dependent on the influx of calcium into the postsynaptic cell. The first demonstration of the calcium dependency for LTP induction came from moderating the amount of calcium entering the cell following depolarisation by altering the extracellular calcium and/or magnesium ion concentrations. Limiting the calcium entry into the cell resulted in the blockade of LTP, but not short-term plasticity (Dunwiddie & Lynch, 1979). Further evidence came a few years later from the same group when it was shown that buffering the intracellular calcium using a calcium chelator completely blocked the induction of LTP (Lynch *et al.*, 1983).

1.3.2.3 NMDA receptor dependent LTP

At around the same time as the discovery of the calcium-dependence of LTP, it was shown that application of *N*-methyl (D, L) aspartate to hippocampal slices results in a depression of synaptic transmission followed by a lasting potentiation of the field EPSP (Collingridge *et al.*, 1983a; 1983b). Furthermore, the induction of LTP (but not the expression of pre-established LTP) is reversibly blocked by the application of the NMDA receptor specific antagonist (D,L)-2-amino-5-phosphonopentanoic acid (AP5, Davies & Watkins, 1982). (N.B. AP5 was formally known as (D,L)-2-amino-5-phosphonovalerate, APV Evans *et al.*, 1977.) The importance of the NMDA receptor in LTP induction has also been demonstrated by drugs that target the

NMDA channel (for example MK-801, Coan *et al.*, 1987) and the allosteric glycine site (for example 7-chlorokynurenic acid, Bashir *et al.*, 1990).

It was noted that the magnesium cation is a potent antagonist of the NMDA glutamate receptor (Coan & Collingridge, 1985; Herron *et al.*, 1985a) and therefore the role of magnesium in the induction process of LTP was investigated by using a Mg^{++}-free medium. This resulted in the generation of epileptiform activity which could then be reversed by the application of D-AP5 (Herron *et al.*, 1985b) indicating that the NMDA receptor is not involved in basal transmission, but suggesting that it may be involved, somehow, in the induction process of LTP. Further evidence came in 1984, when it was shown that the Mg^{++} cation interacts directly with the ion pore of the NMDA receptor and that this interaction is strongly voltage dependent (Mayer *et al.*, 1984; Nowak *et al.*, 1984). These data led to the formation of the NMDA receptor-dependent theory for LTP induction. Activation of AMPA receptors leads to a significant depolarisation within the postsynaptic cell during high-frequency activity to eject the magnesium ion from the NMDA receptor, thus allowing activation of the NMDA channel and calcium influx (Collingridge, 1985). Moreover, the NMDA receptor can be thought of a coincidence detector for both pre- and post-synaptic activity (Collingridge, 2003).

NMDA receptor-dependent LTP has been since been demonstrated in many regions of the cerebral cortex, including visual (Artola & Singer, 1987), perirhinal (Bilkey, 1996), piriform (Jung *et al.*, 1990) and prefrontal (Jay *et al.*, 1995) cortices.

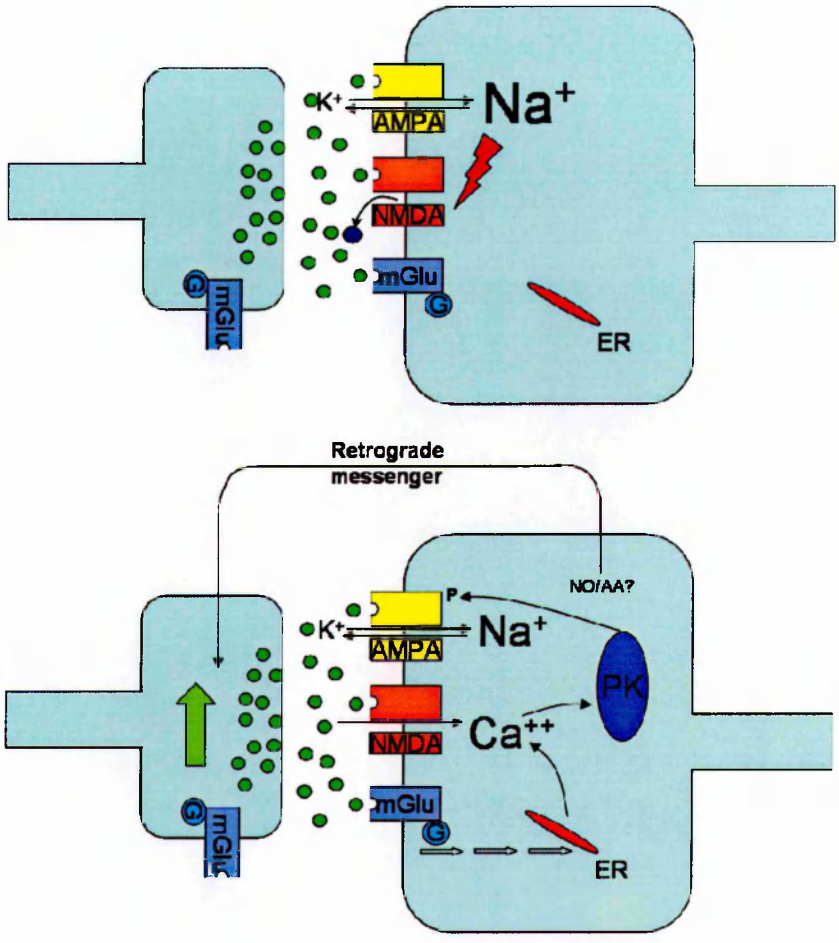


Figure 1.3.4: LTP induction mechanisms. *Top,* High-frequency activity leads to a large influx of sodium, depolarising the cell and ejecting the magnesium ion from the NMDA channel pore. *Bottom*, Calcium can now enter the postsynaptic spine and activate protein kinases, leading to phosphorylation of receptors, which may mediate an increase in responsiveness to activation by glutamate. Generation of retrograde messengers (possibly nitric oxide (NO) and/or arachidonic acid (AA)), may lead to an increase glutamate release upon subsequent presynaptic activity. Alternatively, activation of the metabotropic glutamate receptors may lead to the activation of protein kinases to affect LTD by increasing calcium release from intracellular stores (endoplasmic reticulum, ER); by altering transmitter release; or by other biochemical cascades.

1.3.2.4 NMDA receptor independent/calcium dependent LTP

Interestingly, a form of LTP requiring calcium influx can be induced independently of the NMDA receptor. Very high frequencies of stimulation (200 Hz) have been demonstrated to induce LTP that sources calcium influx through mechanisms other than the NMDA channel. In this form of LTP, calcium enters the postsynaptic cell *via* voltage-sensitive calcium channels (VSCCs, also known as voltage-gated or voltage-dependent calcium channels, Grover & Teyler, 1990). Furthermore, inhibitors of VSCCs also impair NMDA receptor-dependent LTP (Magee & Johnston, 1997), suggesting that calcium influx though the NMDA channel is not the sole source of calcium in NMDA-receptor dependent LTP. Indeed, calcium release from intracellular stores has also been shown to be involved in the raised calcium required for LTP induction. Inhibition of calcium-induced calcium release *via* the ryanodine receptor and/or depletion of calcium stores by thapsigargin both block LTP induction (Obenaus *et al.*, 1989; Harvey & Collingridge, 1992; Bortolotto & Collingridge, 1993).

1.3.2.5 GABA disinhibition

In contrast to the excitatory nature of glutamate, inhibitory systems are also present within the brain. In particular, a class of neurones release γ-aminobutyric acid, which is the major inhibitory neurotransmitter within the CNS. Like glutamate, GABA acts on both ligand-gated ion channels and metabotropic receptors. $GABA_A$ receptors are ionotropic and activation of this receptor leads to influx of chloride

ions, which leads to hyperpolarisation of the cell (see Johnston, 1996 for review). $GABA_B$ receptors are metabotropic in nature and coupled to an inward-rectifying potassium channel and mediate a slow inhibitory postsynaptic potential (see Marshall *et al.*, 1999 for review).

Within the hippocampus there are GABAergic interneurones that receive recurrent collateral axons from glutamatergic neurones and control the excitability and synchronisation of excitatory pyramidal cells (Chapman & Lacaille, 1999). Given the inhibitory nature of the GABAergic system, it is conceivable that LTP could be mediated by a mechanism involving the reduction of inhibition (disinhibition). While several studies suggest that disinhibition does not contribute to CA1 LTP (for example see Haas & Rose, 1982, 1984; Taube & Schwartzkroin, 1988), other studies have demonstrated that stronger intensity conditioning paradigms may indeed reduce the inhibition on hippocampal CA1 pyramidal cells (Misgeld *et al.*, 1979; Miles & Wong, 1987). A mechanism by which disinhibition could be brought about is that of long-term depression of the glutamatergic input onto the interneurones. Such long-term depression has been shown to occur simultaneously with the induction of LTP in the neighbouring CA1 pyramidal cells (McMahon & Kauer, 1997) and may, therefore, play a role in the induction of LTP.

1.3.2.6 mGlu receptor-dependent LTP

In contrast to the calcium-dependent forms of LTP described above, the induction of long-term potentiation can be mediated though metabotropic glutamate receptors. Based on their coupling to signal transduction cascades and their pharmacology there are three groups of mGlu receptor. The three types are made up of subclasses of cloned receptor: Group I comprise mGlu1 and mGlu5; group II comprises of mGlu2 and mGlu3; and mGlu4, mGlu6, mGlu7 and mGlu8 comprise group III. Several splice variants of the mGlu receptors have also been identified. Generally, group I are considered to be positively coupled to the phospholipase C (PLC) cascade, while groups II and III are negatively coupled to adenyl cyclase (AC) and hence the suppression of cAMP production. Other cascades have also been associated with the mGlu receptors and for full reviews see Conn and Pin (1997) and Anwyl (1999). The classification and down-stream signal cascades of mGlu receptors are summarised below in table 1.3.1.

Interest in the role of mGlu receptors in synaptic plasticity came at the end of the 1980s when it was realised that the weak and rather unspecific mGlu receptor antagonists AP3 and AP4 could reduce the duration of LTP in the hippocampus (Reymann & Matthies, 1989; Izumi *et al.*, 1991). These findings have since been confirmed using the relatively more-potent broad-spectrum mGlu receptor antagonist (*RS*)-α-methyl-4-carboxyphenylglycine (MCPG, Sergueeva *et al.*, 1993). Conversely, specific activation of mGlu receptors by (1*S*, 3*R*)-1-aminocyclopentane-1,3 dicarboxylic acid (ACPD) can augment the degree of both short and long-term potentiation obtained in response to a tetanic stimulation (McGuinness *et al.*, 1991).

It has since been demonstrated that several brain regions, including CA1 (Grover & Yan, 1999) and mossy fibre (see Bortolotto *et al.*, 1999) synapses of the hippocampus, the prelimbic (Vickery *et al.*, 1997; Morris *et al.*, 1999; Bindman *et al.*, 2000) and visual (Wang & Daw, 2003) cortices, each support NMDA receptor-independent/mGlu receptor-dependent forms of LTP.

The role of NMDA and mGlu receptors in LTP induction has been found to be mutually exclusive; LTP that requires mGlu receptor activation does not require the activation of NMDA receptors and, conversely, NMDA receptor dependent LTP is mGlu receptor independent (Bortolotto *et al.*, 1999). Although prior mGlu receptor activation may have a priming effect, lowering the threshold for induction, thereby enabling subsequent NMDA receptor activation to more readily induce LTP (Bortolotto *et al.*, 1994). This hypothesis, known as the '*molecular switch*', remains controversial and it is argued that mGlu receptors play only a modulatory role in LTP within the hippocampus (Thomas & O'Dell, 1995).

1.3.2.7 Signal transduction

The influx of calcium into the postsynaptic cell is but the first step in the induction of LTP. Raised calcium concentration within the postsynaptic cell activates various signal transduction cascades including protein kinases, phosphatases, and phospholipases- see figure 1.3.8.

The calcium/phoholipid dependent protein kinase (PKC) was the first protein kinase to be implemented in LTP (Bär *et al.*, 1984; Akers *et al.*, 1986; Lovinger *et al.*, 1986). Inhibitors of this protein kinase invariably prevent the induction and

Class	Subunit	Transduction cascade
Group I	mGluR1	↑PLC→ ↑IP_3→ ↑Ca^{++} release
	mGluR5	
Group II	mGluR2	↓AC→ ↓cAMP→ ↓PKA
	mGluR3	
Group III	mGluR4	
	mGluR6	
	mGluR7	↓AC→ ↓cAMP→ ↓PKA
	mGluR8	

Table 1.3.1: Metabotropic glutamate receptor classification and signal transduction.

maintenance of LTP, even if applied after the tetanus (Lovinger *et al.*, 1987; Malinow *et al.*, 1988; Reymann *et al.*, 1988b). Conversely, injection of the catalytic subunit of PKC into the postsynaptic cell (Hu *et al.*, 1987) or extracellular application of PKC activators (Madison *et al.*, 1986; Malenka *et al.*, 1986) leads to synaptic potentiation.

Another protein kinase involved in LTP is the Ca^{++}/calmodulin-dependent protein kinase type II (CaMKII). An activated, autophosphorylated form of CaMKII has been observed following LTP induction (Barria *et al.*, 1997) and inhibition of this protein kinase prevents the induction of LTP (Mody *et al.*, 1984; Reymann *et al.*, 1988a; Malenka *et al.*, 1989; Malinow *et al.*, 1989; Ito *et al.*, 1991). Transgenic mice studies have further demonstrated the importance of this molecule: Knockout of the gene results in impaired LTP (Silva *et al.*, 1992; Mayford *et al.*, 1996). Additionally, a constitutively active form of the protein kinase induces a lasting form of synaptic potentiation within the hippocampus (Giese *et al.*, 1998).

The other key protein kinase involved in LTP is protein kinase A (PKA). PKA is directly activated in an NMDA receptor-dependent manner within the CA1 region of the hippocampus (Chetkovich & Sweatt, 1993) where it is thought to gate the activity of CaMKII-mediated signal cascades. The calcium that enters the cell *via* the NMDA channel is thought to activate calcium/calmodulin dependent adenyl cyclase, which generates cAMP, in turn leading in the activation of PKA (Chetkovich & Sweatt, 1993; Blitzer *et al.*, 1995). PKA may then phosphorylate inhibitor 1, which inhibits protein phosphatase-1 (PP1) (Huang & Glinsmann, 1976; Blitzer *et al.*, 1998) thereby maintaining the phosphorylation state of CaMKII. This activity is in direct competition with that of protein phosphatase 2B (calcineurin) which dephosphorylates CaMKII (Blitzer *et al.*, 1998). In this scheme, it would

appear that the balance between kinase and phosphatase activity is the crucial determinant in the development of LTP.

PKA also is thought to play a role in late stage LTP. This form of LTP is known to be protein synthesis dependent (Krug *et al.*, 1984; Frey *et al.*, 1988). The activation of PKA in this form of LTP may be *via* the activation of the D_1-like class of dopamine receptors which leads to the activation of adenyl cyclase and hence cAMP production (Frey *et al.*, 1991). Alternatively, mGlu receptor activation may lead to PKA activation though a calcium dependent pathway (Roberson *et al.*, 1999) Once activated, PKA is thought to phosphorylate the cAMP-response element binding protein (CREB, Nguyen *et al.*, 1994), which is known to be increased in a PKA-dependent manner following LTP induction (Impey *et al.*, 1996). Other signal transduction cascades have also been shown to alter the phosphorylation state of CREB, including mitogen-activated protein kinase (MAPK, Impey *et al.*, 1998) and the nitric oxide (NO)- cGMP-dependent protein kinase (PKG, Lu *et al.*, 1999), both of which have been implemented in LTP. It therefore appears that multiple pathways converge on to CREB to produce a long lasting change in protein synthesis (figure 1.3.8).

1.3.2.8 Locus of expression

While the locus of induction is indisputably postsynaptic, the mechanisms involved in expressing and maintaining LTP are a little more contentious. Many arguments have been put forward for both a presynaptic and postsynaptic locus of expression for LTP, with some people favouring the post-synaptic arguments, others the

presynaptic ones. As new evidence arises the arguments for each get stronger, making this a very controversial subject.

There are four feasible mechanisms by which the increase in postsynaptic potential observed following LTP induction could be brought about (see Bliss & Collingridge, 1993):

(i) Presynaptic modifications that give rise to increased neurotransmitter release.

(ii) Postsynaptic changes by which the number of receptors, or the functionality of postsynaptic receptors are increased (or both).

(iii) Extra-synaptic changes, for example altered glia-neurone interactions resulting in an increased neurotransmitter level within the synaptic cleft.

(iv) Morphological changes, including increased number of functional synapses or restructuring of existing synapses.

In reality it is likely that a mixture of all of these changes is involved, each with its own particular different time course.

Evidence for pre- and post- synaptic mechanisms

Initial studies to determine the locus of expression utilised a stimulation protocol known as paired-pulse stimulation, in which two stimuli are applied to the synapse in quick succession. At hippocampal CA1 synapses, this results in an enhancement of the second response with respect to the first, a phenomenon known as paired-pulse facilitation (PPF). PPF is thought to be mediated by a presynaptic mechanism

involving calcium preloading of the synaptic bouton, thereby increasing transmitter release on the second pulse (Zucker, 1973). If LTP were associated with a change in PPF ratios this would be evidence for a presynaptic locus of expression. LTP is not, however, associated with changes in PPF suggesting a postsynaptic locus of expression (McNaughton, 1982; Manabe *et al.*, 1993).

Further evidence for a postsynaptic mechanism comes from studies of AMPA and NMDA receptor-mediated currents before and after LTP induction. If LTP were expressed presynaptically by an increase in transmitter release one would expect to see increases in both AMPA receptor-mediated and NMDA receptor-mediated currents. Following LTP induction AMPA, but not NMDA receptor-mediated components of the EPSP have been shown to be increased, suggesting that potentiation is mediated through changes in the AMPA receptor (Kauer *et al.*, 1988; Muller & Lynch, 1988). Contrary to this, when pharmacologically isolated NMDA receptor components are examined it would appear that NMDA currents do indeed undergo LTP (Bashir *et al.*, 1991; Berretta *et al.*, 1991; Xie *et al.*, 1992; Asztely *et al.*, 1992). This is consistent with a presynaptic increase in transmitter release. Interestingly it has recently been reported in the primary sensory cortex that both AMPA and NMDA currents undergo potentiation, but on a different time scale (Watt *et al.*, 2004). AMPA currents showed a rapid LTP following induction, while NMDA currents only showed an increase at two hours post induction. Furthermore, the increase in NMDA currents was dependent on the AMPA LTP and restored the original NMDA/AMPA ratio. This would indicate that the increase in functional AMPA receptors at the synapse induces a proportional increase in functional NMDA receptors which may be important for maintaining the normal contributions of the receptors to synaptic transmission.

Other experimental approaches that show a postsynaptic locus of expression investigate the sensitivity of neurones following LTP induction. The postsynaptic response to the exogenous agonists AMPA or quisqualate gradually increases following LTP induction (Davies *et al.*, 1989), consistent with an increase in AMPA receptor number or the function of AMPA receptors already present.

A more direct method of assessing locus of expression is that of quantal analysis, which was used in the 1950s by del Castillo and Katz (1954) at he frog neuromuscular junction. The frog neuromuscular junction utilises acetylcholine as its neurotransmitter and is extremely simple compared to the CNS synapse. Del Castillo and Katz noticed that, in the absence of stimulation, small spontaneous postsynaptic potentials occurred, which they termed 'miniature end-plate potentials' (mepp's). Each mepp represents one unit, or 'quantum', of transmitter released from a single vesicle from the presynaptic terminal. The release of each quantum is an all-or-none event under the control of the action potential and has a probability of release (p). The average number of quanta released (m) is obtained by multiplying the number of release sites (n) by 'p'. The release of a single quantum gives rise to a miniature postsynaptic potential of a given size (a), which is determined by the number of postsynaptic receptors. The parameters 'm', 'n', 'p' and 'a' can be estimated by fitting a binomial distribution to frequency histograms of the evoked synaptic potential amplitudes. Such analysis generates a distribution with equally spaced peaks, each peak representing the postsynaptic response to single or multiple fixed quanta and the spaces between peaks is equal to the quantal size. Variance of the mean synaptic potential amplitude is termed the 'coefficient of variance' and is inversely proportional to the quantal content.

The formidable task of applying quantal analysis to central synapses to investigate changes following LTP induction has been attempted. Many problems are associated with this including variability in synapse size and shape, receptor distributions and non-fixed release probabilities. These problems are compounded by the fact that a single neurone receives inputs from maybe thousands of synapses, of which only a few may exhibit LTP. To circumvent these problems both minimal stimulation (in an attempt to achieve miniature postsynaptic potentials) and pharmacology (replacement of calcium with strontium ions to cause asynchronous release at activated synapses only) have been used.

LTP at central synapses has been associated with a decrease in the coefficient of variance and also in the failure rate (Malinow & Tsien, 1990; Bekkers & Stevens, 1990; Kullmann & Nicoll, 1992; Manabe *et al.*, 1993). Both of these changes suggest a presynaptic mechanism of expression.

Presynaptic mechanisms of expression had previously been suggested. The most direct evidence coming from measurements of neurotransmitter release prior to and following LTP induction. The first of such experiments showed that release of radio-labelled glutamate increases following LTP induction in the dentate gyrus (Dolphin *et al.*, 1982). Furthermore, endogenous glutamate levels, as measured by high pressure liquid chromatography following *in vivo* sampling from a push-pull cannula, are increased after LTP induction in the dentate gyrus (Bliss *et al.*, 1986; Errington *et al.*, 1987; Lynch *et al.*, 1989) and CA1 (Ghijsen *et al.*, 1992). In addition, these increases are dependent on NMDA receptor activation and are specific to glutamate (Errington *et al.*, 1987). Depolarisation-induced glutamate release is also enhanced following LTP induction (Skrede & Malthe-Sorenssen, 1981; Errington *et al.*, 1987; Canevari *et al.*, 1994). The importance of the evidence

for a presynaptic mechanism of expression is underlined by the fact that similar enhancements in glutamate release are observed following hippocampal-dependant learning in the Morris water maze (Richter-Levin *et al.*, 1995, 1998).

Postsynaptic mechanisms

The simplest model for a postsynaptic mechanism of LTP expression is of AMPA receptor modification. Changes in AMPA receptor function could be brought about by direct phosphorylation by protein kinases activated by LTP induction. The potent protein kinase inhibitor K-252b prevents the increase in AMPA receptor sensitivity following LTP induction (Reymann *et al.*, 1990). Conversely, addition of the PKA catalytic subunit can directly increase AMPA receptor conductance (Greengard *et al.*, 1991; Wang *et al.*, 1991).

An alternative model is based on the finding that AMPA receptors exist in two distinct isoforms each with different conductance properties, termed 'flip' and 'flop', whereby the relative expressions of the two could change following LTP induction (Sommer *et al.*, 1990). There could also be a change in the general expression of the cloned AMPA subunits GluR1-4 (Gasic & Hollmann, 1992).

Silent synapses

In light of the apparent increase in glutamate release highlighted by quantal analysis, a postsynaptic mechanism (rather than the obvious presynaptic ones) was proposed in which the number of functional synapses increases following LTP. This theory has been termed the 'silent synapse' and describes the presence of electronically inactive synapses existing under basal conditions (Kullmann, 1994). This could be achieved by the absence of AMPA receptors at a synapse where NMDA receptors are expressed. Such a receptor distribution would result in a silent synapse, as the NMDA receptor is inactive at resting membrane potential. The insertion of AMPA receptors into this synapse following LTP would un-silence the synapse, thereby converting it into a functional one (see figure 1.3.5). Such a mechanism fits both the apparent increase in transmitter release indicted by quantal analysis (which is assessed using the postsynaptic response) and the increase in AMPA receptor-mediated currents (see Kullmann, 2003 for review.)

Silent synapses have been demonstrated electrophysiologically by two independent research groups (Isaac *et al.*, 1995; Liao *et al.*, 1995). These synapses showed no discernable AMPA receptor-mediated currents, but had normal NMDA channel-mediated currents in response to minimal stimulation. Following LTP induction AMPA components were detectable. These results have also been correlated with immunocytochemical studies. While virtually all excitatory synapses contain NMDA receptors, only a portion of synapses stain for AMPA receptors (Rao & Craig, 1997; Gomperts *et al.*, 1998; Liao *et al.*, 1999), a result confirmed at the ultrastructural level (Nusser *et al.*, 1998; Petralia *et al.*, 1999).

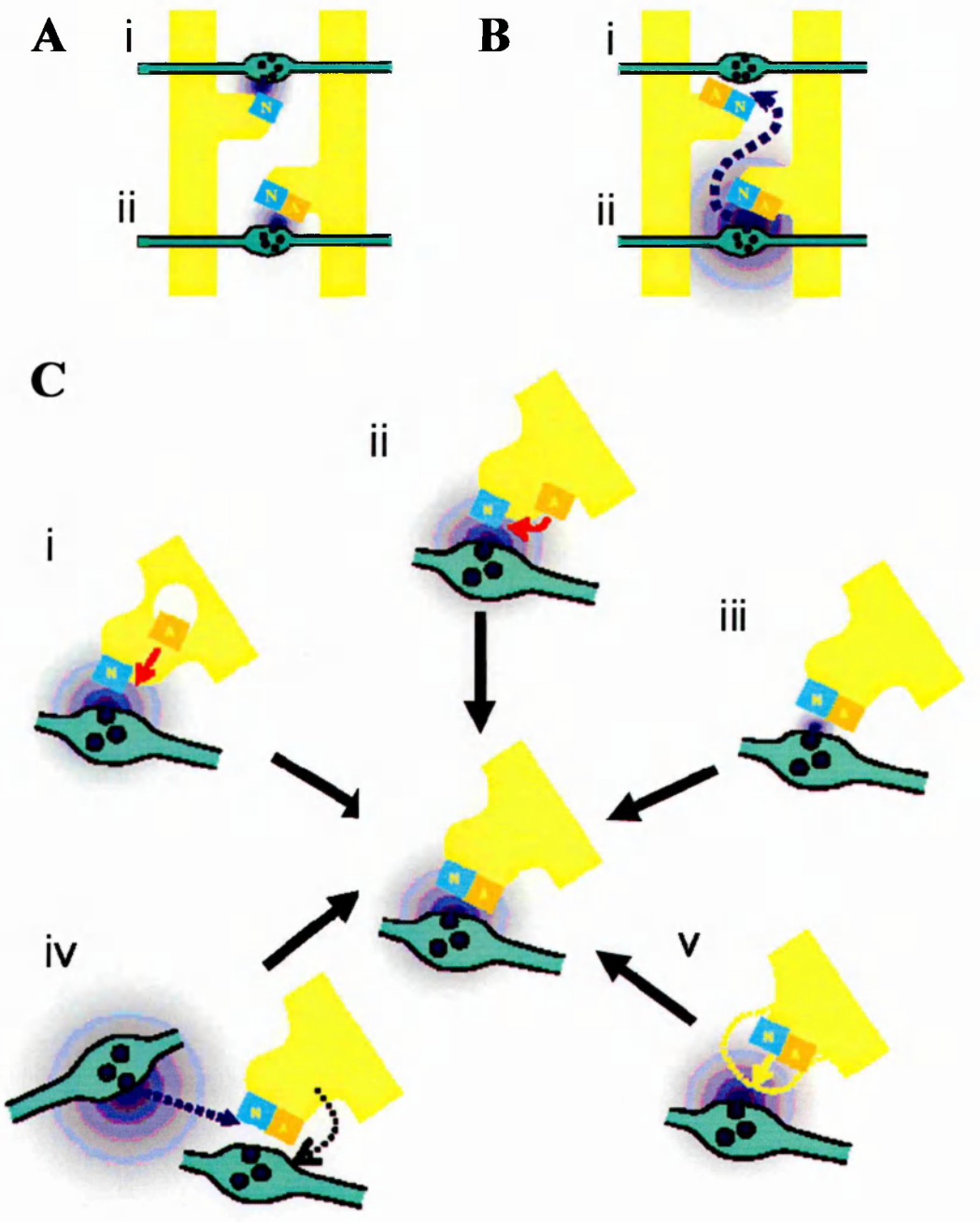


Figure 1.3.5: Silent synapses. *A*, synapse i expresses only NMDA receptors and no functional AMPA receptors, making this a silent synapse. In contrast, synapse ii expresses both receptors, making this a functional synapse. *B*, Glutamate spill-over activates only NMDA receptors in a presynaptically inactive synapse. The releasing varicosity (ii) is likely to be in synaptic contact with a different neurone (i), explaining why no AMPA-mediated component is detected. *C*, Un-silenceing by LTP could be mediated by different mechanisms. i) AMPA receptor insertion into the synapse; ii) movement of perisynaptic AMPA receptors to the synaptic cleft; iii) change in the release probability of glutamate from the presynaptic terminal; iv) conversion of a presynaptically inactive synapse to an active one triggered by activity of neighbouring synapses; v) structural changes of the postsynaptic spine could lead to its approach to a release site. (Adapted from Kullmann, 2003.)

An alternative mechanism to that of un-silencing by AMPA receptor insertion could be glutamate spill-over (see Kullmann & Asztely, 1998, figure 1.3.5). In this model, high levels of glutamate released from a group of synapses could activate NMDA receptors in the AMPA-absent synapses. This mechanism is not purely a postsynaptic mechanism, as it requires an increase in glutamate release for the activation of receptors outside the synaptic cleft of the activated synapse. Equally this mechanism is no longer strictly homosynaptic.

Another mechanism of un-silencing may involve growth of synaptic spines, thereby reducing the distance between pre- and post-synaptic membranes. The proposed mechanisms of un-silencing are summarised in figure 1.3.5.

Presynaptic mechanisms

Although the mechanism of induction is indisputably postsynaptic there is significant evidence for both a pre- and post-synaptic locus of expression. To reconcile this apparent discrepancy between a postsynaptic induction and a presynaptic expression it has been proposed that an intracellular signal is released from the postsynaptic cell to initiate the increase in release of neurotransmitter from the presynaptic terminal (see figure 1.3.4).

In order for a molecule to act as a retrograde messenger it must meet a number of criteria (see Bliss & Collingridge, 1993): 1, it must have a postsynaptic synthesis that is amplified following LTP induction; 2, it must be released into the synaptic cleft to diffuse into the presynaptic terminal; 3, it must affect increased transmitter release. Several studies have provided potential candidates for the retrograde messenger including peptides (Duffy *et al.*, 1981; Fazeli *et al.*, 1988;

Otani *et al.*, 1992), arachidonic acid (see below) and nitric oxide (NO; discussed below). Peptides were soon dismissed on the basis that their onset was too slow to cause the presynaptic change. In contrast both arachidonic acid and NO have been considered suitable candidates.

Arachidonic acid

Arachidonate is synthesised by the enzyme phospholipase A_2, inhibiters of which block the induction of LTP (Okada *et al.*, 1989; Williams & Bliss, 1989). Conversely, the transient application of arachidonate to hippocampal slices induces a slow-onset potentiation (Barbour *et al.*, 1989; Williams *et al.*, 1989). It has been shown to be released into the extracellular matrix following activation of the NMDA receptor (Dumuis *et al.*, 1988) and shows increased efflux and postsynaptic availability following LTP induction (Williams *et al.*, 1989). Targets of arachidonic acid are both the presynaptic terminal, where it increases glutamate release (Williams *et al.*, 1989) and glia, where it suppresses glutamate uptake (Barbour *et al.*, 1989).

Nitric oxide

The other candidate for the retrograde messenger is nitric oxide. NO is a membrane-permeable gas that is synthesised from L-arginine by the calcium/calmodulin-dependent enzyme nitric oxide synthetase (NOS). CA1 pyramidal cells express NOS and, moreover, release NO gas following NMDA receptor activation (Schuman & Madison, 1994). Pharmacological inhibition of NOS (Schuman & Madison, 1991), haemoglobin scavenging of NO (Arancio *et al.*, 1996) and gene knock-out of NOS

(O'Dell *et al.*, 1994; Son *et al.*, 1996) all prevent the induction of LTP. Furthermore, photolytic release of NO from caged compounds, paired with simultaneous afferent stimulation, induces potentiation (Arancio *et al.*, 1996).

Contradictory, however, some groups have failed to replicate NO-induced LTP (for example see Haley *et al.*, 1993 and; Cummings *et al.*, 1994), that blockade of LTP by NOS inhibitors was temperature- and age-dependent (Williams *et al.*, 1993) and, furthermore, that NO could actually depress NMDA receptor-mediated currents (Murphy *et al.*, 1994; Murphy & Bliss, 1999). These findings suggest that NO may modulate the NMDA receptor to raise the threshold for subsequent LTP induction.

1.3.3 Long-term depression

In contrast to long-term potentiation, long-lasting decreases in synaptic transmission can also be induced at central synapses (see figure 1.3.6). The idea that synaptic connections could undergo decremental changes was proposed by Gunther Stent who suggested a complementary postulate to that of Donald Hebb: Synapses that are inactive during periods of activity in the postsynaptic neurone could undergo a process by which synaptic transmission is weakened (Stent, 1973). Long-lasting synaptic depression was identified in the late 1970s, when it was found that a reversible depression was induced in the non-tetanised pathway during LTP induction within the CA1 region of hippocampal slices (Lynch *et al.*, 1977). These decreases in synaptic transmission were termed *heterosynaptic depression* and has also been demonstrated *in vivo* within the dentate gyrus (Levy & Steward, 1979).

Depression of previously potentiated synaptic responses has also been shown, termed depotentiation (Hesse & Teyler, 1976). Identification of *homosynaptic* long-term depression of naïve synapses was, however, elusive and the phenomenon of depression was largely ignored until the early 1990s. *De novo* homosynaptic LTD was eventually identified in response to a low-frequency conditioning stimulus applied to CA1 synapses of hippocampal slices prepared from young rats (Dudek & Bear, 1992; Mulkey & Malenka, 1992). The input-specific nature of LTD is an important observation as it excludes the attribution of the decreased synaptic transmission to a general decline in slice health. Furthermore, homosynaptic LTD, like LTP, fits many of the requirements for a mechanism underlying learning and memory.

LTD within the hippocampus was found to be developmentally down-regulated and by approximately 35 days, LFS was no longer effective at inducing LTD in the rat. A similar down regulation of LTD has recently been demonstrated in the mouse hippocampus (Milner *et al.*, 2002a; 2003b; 2004b). The down regulation of LTD observed in the rat has been shown to be overcome by using an enhanced LFS protocol. At ages where standard LFS fails to induce depression (12-16 weeks) a train of paired-pulse stimuli has been found effective at inducing LTD (Kemp & Bashir, 1997).

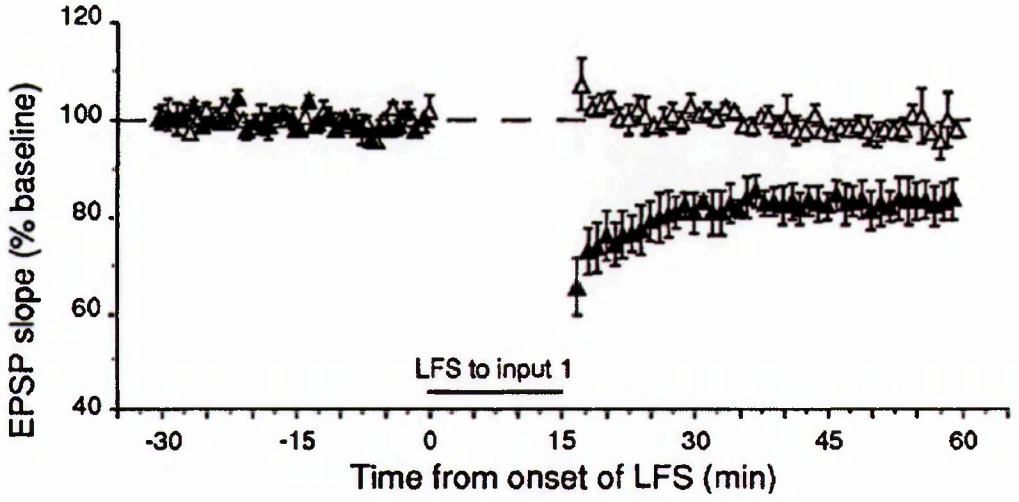


Figure 1.3.6: The first demonstration of homosynaptic LTD. Low-frequency stimulation (LFS) applied to the experimental pathway (▲) results in a long-lasting depression in that pathway, but not the control pathway (Δ) which does not receive LFS. (Adapted from Dudek & Bear, 1992.)

It has been established that many regions of the forebrain also support homosynaptic LTD including striatum (Calabresi *et al.*, 2000; Spencer & Murphy, 2000), visual cortex (Artola *et al.*, 1990), prefrontal cortex (Hirsch & Crepel, 1990), amygdala (Wang & Gean, 1999) and perirhinal cortex (Cho *et al.*, 2000). Importantly, LTD in these regions is not developmental and persists into adulthood using standard LFS protocols.

1.3.3.1 NMDA receptor-dependent LTD

The initial studies carried out in CA1 of the hippocampus demonstrated that LTD was dependent on the activation of NMDA receptors (Dudek & Bear, 1992; Mulkey & Malenka, 1992) and this finding has subsequently been supported by other studies (Kemp & Bashir, 1997; Kemp *et al.*, 2000; Milner *et al.*, 2004b). Direct application of NMDA has also been demonstrated to induce CA1 LTD with similar properties to that induced by LFS, in that it is down-regulated with age, is reversible and saturable (Lee *et al.*, 1998). The down-regulation of NMDA receptor-dependent LTD has been proposed to be mediated by an age-dependent shift in NMDA receptor subunit expression from NR2B to NR2A (Insel *et al.*, 1990; Monyer *et al.*, 1994). Contrary to this view, however, mice over expressing the NR2B subunit into adulthood do not show facilitated LTD (Tang *et al.*, 1999).

NMDA receptor-dependent, homosynaptic LTD has also been demonstrated within the dentate gyrus of intact animals (Thiels *et al.*, 1994; Heynen *et al.*, 1996). Within the cortex several reports have demonstrated NMDA-receptor dependent LTD, including visual (Kirkwood & Bear, 1994; Sawtell *et al.*, 1999), somatosensory

(Feldman *et al.*, 1998) and perirhinal (Cho *et al.*, 2000, perirhinal cortical plasticity will be discussed in section 1.5) cortices. Generally, LTD that is dependent on NMDA receptor activation is independent of the mGlu receptor subclass (for example see Kemp & Bashir, 1997; Sawtell *et al.*, 1999; Milner *et al.*, 2004b; but see Wang & Gean, 1999; Cho *et al.*, 2000).

1.3.3.2 mGlu receptors and LTD

In contrast to NMDA receptor dependent LTD there are many examples of LTD induction that is dependent on the activation of metabotropic glutamate receptors. Within the hippocampus the broad-spectrum mGlu receptor antagonist MCPG blocks several forms of LTD (for example see Manahan-Vaughan, 1997; Oliet *et al.*, 1997; Otani & Connor, 1998; Bortolotto *et al.*, 1999). The mechanisms involved in mGlu receptor-mediated LTD are likely to be different than those of NMDA receptor-dependent LTD because saturation of one type does not occlude the induction of the other (Palmer *et al.*, 1997; Oliet *et al.*, 1997)

Metabotropic glutamate receptor-dependent LTD has also been demonstrated in cortical regions including prefrontal cortex (Otani *et al.*, 1999), perirhinal cortex (see section 1.5) and between interneurones of the somatosensory barrel cortex (Egger *et al.*, 1999).

1.3.3.3 LTP or LTD?

The induction mechanism for both LTP and LTD is an increase in postsynaptic calcium. How does this common signal confer bi-directional plasticity?

Donald Hebb (1949), in the first postulate of activity-dependent facilitation of synaptic transmission, predicted that there is an activity threshold for increases to occur. Conversely, Gunther Stent (1973) proposed that synapses that are inactive during postsynaptic activity might undergo a weakening process. In a conglomerate theory, it was proposed that the direction of plasticity is correlated with the degree of activity, such that active synapses are strengthened when a 'modification threshold' (θ_m) is exceeded (same as Hebb's proposal), and (in contrast to Stent) that depression only occurs at *active* synapses when the postsynaptic activity is less than θ_m but higher than a lower threshold (Cooper *et al.*, 1979). An addition to this theory was later added that previously integrated postsynaptic activity can alter the value of θ_m (Bienenstock, Cooper and Munro, 1982, BCM theory, see figure 1.3.7).

A demonstration of the BCM theory was provided by differential induction of LTP or LTD by application of the same high-frequency stimulation protocol to neurones of the rat visual cortex. The direction of plasticity was found to be dependent on the degree of depolarisation achieved in the postsynaptic cell, with higher degrees of depolarisation leading to LTP and lower degrees to LTD (Artola *et al.*, 1990).

The BCM theory came to the fore when it was realised that both LTP and LTD are calcium dependent. It was then proposed that input activity giving rise to calcium concentrations exceeding θ_m would result in LTP, while calcium

concentrations lower than θ_m, but exceeding a lower threshold, would induce LTD. Furthermore, the upper threshold could vary depending on the previous activity at the synapse which could alter NMDA receptor function (Bear *et al.*, 1987). Similar proposals have been made by Lisman (1989) and Artola and Singer (1993)

Direct evidence for distinct calcium thresholds for LTP or LTD induction was provided by fluorescence measurements of calcium transients following glutamate ionophoresis to induce plastic changes in CA1 hippocampal neurones (Cormier *et al.*, 2001). It was found that discrete ranges of calcium levels correlate with either LTP or LTD induction. Below ~180 nM calcium, no plasticity was observed; between ~180 and ~450 nM LTD was induced; another aplastic range was found between ~450 and 540 nM; levels above ~540 nM led to LTP; a finding that fits the BCM theory exactly (see figure 1.3.7).

The above description is probably an over simplification, as both spatial and temporal considerations, including decay time; distal or proximal sources of calcium; and duration of activity are likely to play important roles. For instance a protracted duration of activity and calcium influx is needed for the induction of LTD, while a shorter burst of activity is required for LTP (Mizuno *et al.*, 2001)

1.3.3.4 Signal transduction

The mechanisms involved in the expression of LTD are less understood than those of LTP. Some of the biochemical cascades involved have been identified and are discussed here and are summarised in figure 1.3.8.

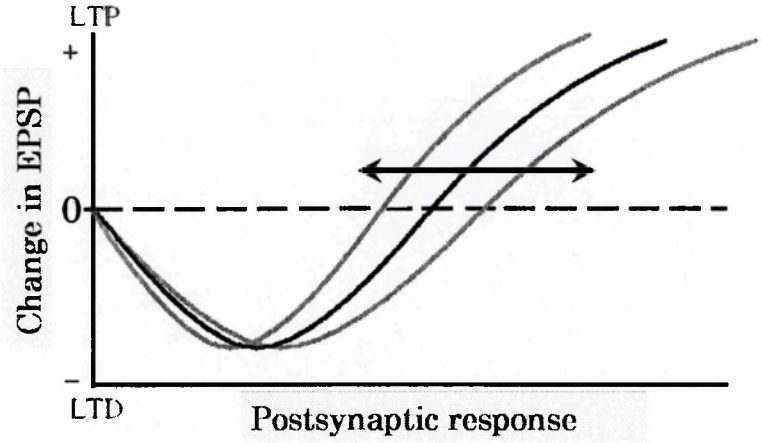


Figure 1.3.7: The BCM theory of bidirectional plasticity. The degree of activity achieved in and hence the degree of calcium entering, the postsynaptic cell during conditioning determines the direction of plasticity (black curve). Previous activity at a synapse may modify the threshold for the induction of LTD or LTP (grey curves). (Based on figures in Bear, 2003.)

Calcium dependent biochemical cascades

The different calcium signals achieved during LTP and LTD induction could confer differential activity of protein kinases and protein phosphatases. The short, large increase associated with LTP will activate CaMKII and the associated cascades mentioned in section 1.3.3, while a prolonged smaller calcium signal will activate protein phosphatase 2B: calcineurin (proposed by Lisman, 1989). While CaMKII will lead to phosphorylation of AMPA receptors (Barria *et al.*, 1997) and mediate LTP (Lledo *et al.*, 1995), activation of protein phosphatase 2B will dephosphorylate and thereby activate inhibitor 1, in turn activating protein phosphatases 1 and 2 resulting in dephosphorylation of AMPA receptors (see Kemp & Bashir, 2001 for review).

Similarly to LTP, other cascades are implicated in LTD. The phospholipase A_2 pathway, in a dose dependent manner, is thought to alter AMPA receptor affinity states to enable LTP/LTD expression (Chabot *et al.*, 1998). The cGMP/NO pathway is potentially involved, with exogenous application of NO inducing a long-lasting depression (Izumi & Zorumski, 1993; Murphy *et al.*, 1994; Murphy & Bliss, 1999).

G-protein linked pathways

Activation of the metabotropic class of glutamate receptors is linked to a variety of pathways. Group I mGlu receptors are associated with the activation of PLC which can 1) affect release of calcium from internal stores through the IP_3 pathway (see Conn & Pin, 1997) and 2) increase PKC levels leading to CREB-dependent protein synthesis via the MAP kinase pathway (Fiore *et al.*, 1993; Orban *et al.*, 1999).

Interestingly the levels of MAP kinases have been shown to be decreased following LTD induction (Norman *et al.*, 2000).

Group II mGlu receptors are negatively coupled to the production of cAMP, thereby suppressing PKA-dependent phosphorylation of AMPA receptors (Kameyama *et al.*, 1998; Tzounopoulos *et al.*, 1998). Conversely, inhibition of PKA blocks LTD induction (Huang & Glinsmann, 1976), a finding consistent with the observation that mice lacking the PKA catalytic subunit or regulatory subunit are deficient in LTD (Brandon *et al.*, 1995; Qi *et al.*, 1996). These contradictory findings could be explained by a basal level of PKA activity governing the phosphorylation state of AMPA receptors, thereby either increases or decreases in PKA activity can effect synaptic transmission.

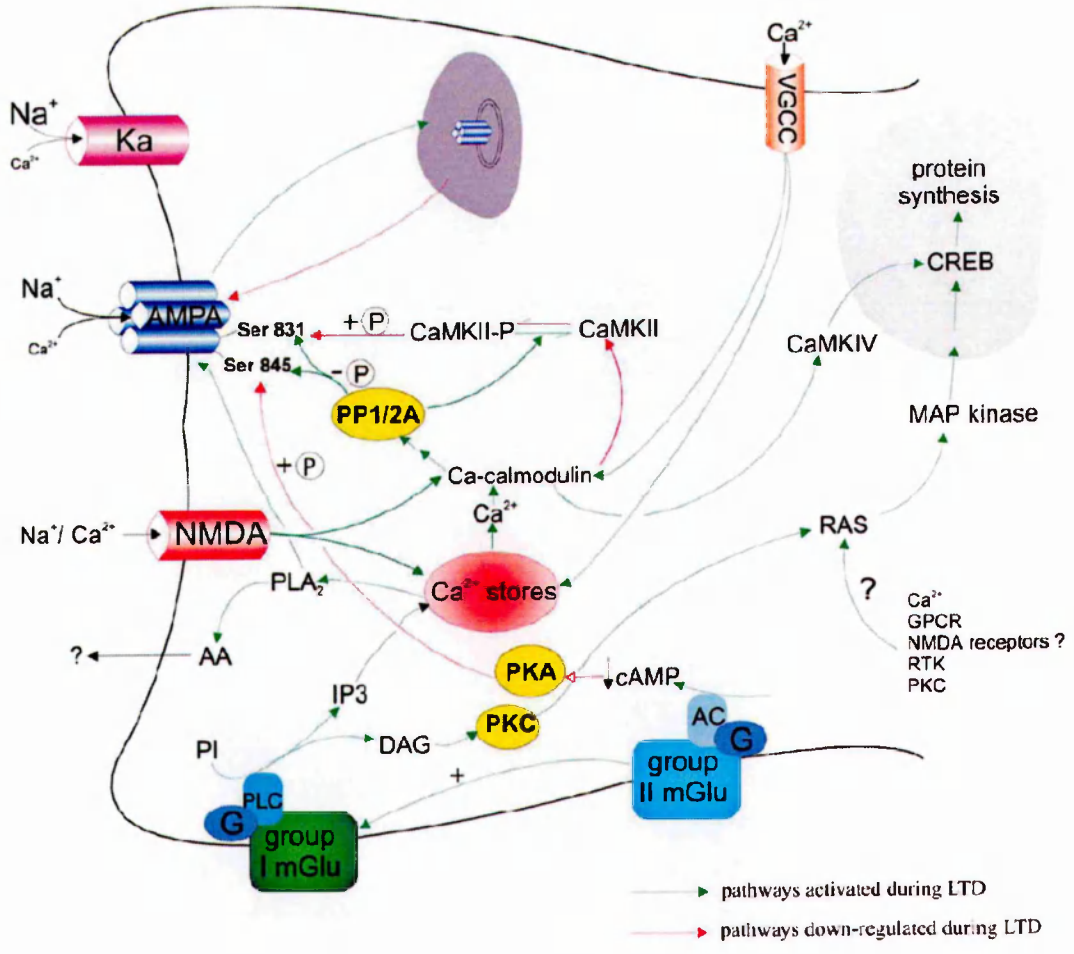


Figure 1.3.8: Schematic illustration of some of the biochemical cascades involved in synaptic plasticity. Abbreviations: AC, adenylate cyclase; AA, arachidonic acid; CaMKII, calcium–calmodulin dependent protein kinase II; CREB, cAMP response element binding protein; DAG, diacylglycerol; IP3, inositol triphosphate; KA, kainate receptor; mGlu, metabotropic glutamate receptor; MAP kinase, mitogen-activated protein kinase; PI, phosphatidyl inositol; PLC, phospholipase C, PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PP1/2A, protein phosphatase 1/2A; and TKR, tyrosine kinase receptor. (Taken from Kemp & Bashir, 2001.)

Group III mGlu receptors are also negatively coupled to adenyl cyclase and the production of cAMP. Until recently a role for type III mGlu receptors in LTD had not been demonstrated (for examples see Palmer *et al.*, 1997; Fitzjohn *et al.*, 1999; Cho *et al.*, 2000; Mellentin & Abraham, 2001). An article published this year has shown that inhibition of group III mGlu receptors by (RS)-a-cyclopropyl-4-phosphonophenylglycine (CPPG) inhibits the induction of LTD in the dentate gyrus of freely moving rats (Klausnitzer *et al.*, 2004).

1.3.3.5 Expression of LTD

The biochemical cascades involved in LTD suggest that alterations in the function of AMPA receptors could underlie the depression of synaptic transmission. Another mechanism that has been proposed is that of silencing synapses; the converse of the mechanism proposed for LTP. LTD in cell cultures has been associated with a decrease in surface expression of AMPA receptors (Carroll *et al.*, 1999b) and also exposure to low levels of glutamate can decrease the ratio of GluR1 to NR1 subunits (Lissin *et al.*, 1998). The mechanism by which synapses are silenced could involve endocytosis of AMPA receptors. Disruption of dynamin-dependent endocytosis could lead to an increase in AMPA-mediated currents (Carroll *et al.*, 1999a; 1999b). Conversely, application of insulin induces endocytosis, leads to decreased postsynaptic responses and induces LTD that occludes further LFS-induced LTD. Furthermore, restricting endocytosis precludes LTD induction (Man *et al.*, 2000).

Presynaptic changes are also likely to occur and, as with LTP, a mixture of both loci is probable. Evidence for a presynaptic mechanism is provided by studies

of failure rate, quantal size, coefficient of variance and number of vesicles available for release (for examples see Bolshakov & Siegelbaum, 1994; Stevens & Wang, 1994; Goda & Stevens, 1998; Lüthi *et al.*, 1999).

Similarly to LTP expression, it is likely that LTD involved a mixture of both pre- and postsynaptic alterations.

1.4 Synaptic plasticity in Huntington's disease

As highlighted in section 1.3, cognitive decline is the earliest manifestation of Huntington's disease, seen long before evidence of cellular pathology and neuronal death; and full dementia is a classical hallmark of the later stages. Similarly, transgenic mouse models show deficits on learning tasks, particularly in the hippocampal-dependent test of spatial memory the Morris water maze (Lione *et al.*, 1999; Murphy *et al.*, 2000). Given that cognitive processes are encoded within the brain by changes in synaptic transmission, synaptic plasticity has been investigated within these mice.

The electrophysiology of two brain regions has been investigated: The striatum, being the region showing greatest susceptibility to neurodegeneration; and, in light of the deficits in hippocampal-dependent learning tasks, the hippocampus. In contrast there are no extensive reports on synaptic transmission or plasticity within cortical regions of any transgenic mouse models of HD.

1.4.1 Striatum

The basal ganglia are particularly susceptible to neurodegeneration during the progress of HD. In particular there is a selective loss of MSSNs in the striatum (Vonsattel *et al.*, 1985). Similarly, in the R6 mouse lines, a selective, late-onset neurodegeneration is observed within the dorsal striatum (Turmaine *et al.*, 2000). There is growing evidence, however, that neuronal dysfunction predates the actual

loss of cells, suggesting that impairments in subcortical cognition may occur in the absence of neuronal death. The principle input to the striatum is from the cortex and the corticostriatal pathway in several mouse lines has received extensive investigation.

1.4.1.1 Basal cellular properties

Several studies have shown MSSNs to have a depolarised resting membrane potential. In the R6/2 mouse the majority of MSSNs have been found to be depolarised in the resting state within symptomatic, but not presymptomatic mice (Levine *et al.*, 1999; Klapstein *et al.*, 2001). In contrast a knock-in mouse with 100 glutamine repeats has MSSNs with normal membrane potentials (Laforet *et al.*, 2001). Interestingly, another knock-in mouse, with either 71 or 94 CAG repeats, has a subpopulation of MSSN cells exhibiting depolarisation at rest (Levine *et al.*, 1999). In the 94Q mouse approximately 40% of MSSNs are depolarised, while in the 71Q mouse 20% show a depolarisation. A depolarised resting membrane potential could be a result of impaired energy metabolism which has been reported in HD (see section 1.2.7.1). Reduced ATP availability would decrease the activity of Na^+/K^+/ATPase that is crucial in maintaining the intracellular concentrations of potassium and sodium ions, the principle determinants of membrane potential.

The input resistance of MSSNs has been reported to be increased in both symptomatic and presymptomatic R6/2 mice (Levine *et al.*, 1999; Klapstein *et al.*, 2001). Again in contrast to R6/2 mice, the input resistance of MSSNs from the

HD100 knock-in mouse show a reduced input resistance (Laforet *et al.*, 2001), while the 71 and 94 repeat mice show no change (Levine *et al.*, 1999).

With the exception of a reduced action potential amplitude in MSSN cells of symptomatic R6/2 mice, most aspects of the action potential (including threshold and duration) have been reported as normal in all mouse lines studied (Levine *et al.*, 1999; Klapstein *et al.*, 2001; Laforet *et al.*, 2001).

1.4.1.2 Basal synaptic transmission

The frequency of spontaneous EPSPs recorded from MSSNs is reduced in R6/2 at 5-7 weeks of age and this reduction is more pronounced at 11-15 weeks. At 3-4 weeks, however, spontaneous activity is similar to wild-types. There is also an increased incidence of large synaptic events in R6/2 at the later ages (Cepeda *et al.*, 2003). Similar reductions in spontaneous activity are also seen in the R6/1 mouse (Cepeda *et al.*, 2003).

The size of evoked EPSPs are smaller in both R6/2 and HD100 knock in mice (Klapstein *et al.*, 2001; Laforet *et al.*, 2001). Furthermore, the stimulus intensity required to evoke an action potential in both presymptomatic and phenotypic R6/2 mice is increased (Klapstein *et al.*, 2001). These results may be indicative of less neurotransmitter release, or reduced postsynaptic responsiveness. In support of a presynaptic change, paired-pulse facilitation is reduced in both presymptomatic and symptomatic mice (Klapstein *et al.*, 2001).

In support of postsynaptic changes, the NMDA receptor has been shown to have enhanced function. Currents induced by NMDA application show increases in the R6/2, both knock-in mice studied and the YAC72 model (Cepeda *et al.*, 2001; Zeron *et al.*, 2002). This may be a direct result of the depolarised state of the neurones, however when cells were held at standardised holding potentials, NMDA current densities were still enhanced in mutant mice over those of wild-types. Furthermore, calcium flux in response to NMDA is increased (Laforet *et al.*, 2001). The increased degree of calcium entering the cell is likely to underlie the enhanced NMDA-induced cell swelling observed by Levine *et al.* (1999).

The enhancement in NMDA receptor function by mutant huntingtin appears to be subtype-specific. Coexpression of either normal or mutant huntingtin, with NR1 and either NR2A or NR2B subunits in HEK293 cells highlighted subclass specific enhancements in NMDA-mediated currents (Chen *et al.*, 1999b). Coexpression of the NR2B subunit with mutant huntingtin results in an increased NMDA receptor-mediated current above that seen when normal huntingtin is coexpressed. In contrast, no such increases were seen with the NR2A subunit with either normal huntingtin or a control protein (β-gal). Similarly, NMDA mediated excitotoxicity is enhanced in a subtype specific manner within acutely dissociated MSSNs from YAC72 mice (Zeron *et al.*, 2001) and this enhancement is reduced by the application of the NR2B subtype specific antagonist Ifenprodil (Zeron *et al.*, 2002).

1.4.1.3 Synaptic plasticity

The corticostriatal pathway provides the main glutamatergic inputs to the MSSNs of the striatum and corticostriatal synapses are known to support both LTP and LTD (Calabresi *et al.*, 2000; Spencer & Murphy, 2000). No published data is available on synaptic plasticity within the corticostriatal pathway of HD mice. Preliminary data from within our lab suggests that LTP is enhanced in the R6/2 mouse (see Bates & Murphy, 2002) and this may be due to the increased NMDA function.

1.4.2 Hippocampus

The most comprehensive characterisation of synaptic plasticity in mouse models of HD is within the hippocampus. Crucially R6 mice are impaired in hippocampal-dependent spatial learning tasks prior to an overt phenotype (Murphy *et al.*, 2000). Three HD mouse models have been reported with abnormal synaptic plasticity within the hippocampus: a knock-in with 72 and 80 CAG repeats, the YAC full length transgenic and the R6/2 mouse. I will first describe the data from each mouse and then discuss the common and divergent findings.

1.4.2.1 HD72 and 80 knock-in

The first report of long-term synaptic plasticity in the hippocampus was within the Shelbourne knock-in mouse (Usdin *et al.*, 1999). One will recall that the only

reported behavioural phenotype in this mouse was increased aggression (which is probably attributable to other causes then the CAG repeat *per se*) and is therefore a model of the early stages of the disorder.

Basal transmission at CA1 synapses was found to be normal, as demonstrated by input-output curves from mutant and wild-type mice being identical.

Long-term potentiation was induced by high-frequency stimulation. LTP was found to be impaired in the majority of transgenic slices compared to that recorded in slices from wild-type animals. Because some slices exhibited apparently normal LTP the authors proposed that the mechanism for induction may be functional, but that the threshold for induction is increased. Indeed, when a stronger tetanus was applied, LTP was indistinguishable from that recorded in slices from wild-type mice.

Post-tetanic potentiation (PTP) was, however, still impaired. As PTP is an indicator of presynaptic function, Usdin and co-workers predicted a presynaptic mechanism for this impairment, a proposal confirmed by reduced paired-pulse facilitation. To test this hypothesis further the properties of the activity-dependent NMDA channel blocker MK801 was used to assess transmitter release. The rate of decay of NMDA receptor-mediated currents is directly proportional to the amount of transmitter released. At low-frequency stimulation (basal transmission) there was no difference between the decay rate at HD and wild-type synapses, while at high-frequency, NMDA currents showed a slower rate of decline in mutant mice (Usdin *et al.*, 1999). This was interpreted as an impaired ability to sustain transmitter release under high-frequency rather than an increased induction threshold as initially proposed.

1.4.2.2 YAC transgenics

The second report of abnormal synaptic plasticity in HD was within the YAC mouse expressing the entire human HD gene with either 46 or 72 CAG repeats (Hodgson *et al.*, 1999). It should be noted that these experiments were carried out in artificial cerebrospinal fluid (ACSF) that lacked the magnesium ion, which would normally regulate NMDA receptor activity.

Repetitive tetanic stimulation was used to induce saturated LTP at CA3-CA1 synapses. At six months old both YAC46 and YAC72 mice (both presymptomatic) show normal LTP. By ten month the YAC 46 mice (still aphenotypic) completely failed to express LTP and the YAC72 mice (phenotypic) display a slow onset, homosynaptic LTD in response to tetanic stimulation.

At six months, slices prepared from YAC72 mice showed hyperexcitability that was sensitive to the NMDA receptor antagonist D-AP5. The hyperexcitability was not, however, reported as a comparison to wild type or transgenic mice at other ages or carrying different repeats. Given that magnesium-free ACSF is known to induce NMDA receptor-dependent epileptiform activity (Herron *et al.*, 1985b), this evaluation is an important control experiment that was not reported.

Slices prepared from the YAC46 mice showed decreased glutamate-induced calcium currents. Decreased calcium currents could be mediated by less NMDA receptors or decreased NMDA receptor function and this appears to be contradictory to their claim of increased NMDA receptor activity. It was found, however, that the suppressed calcium influx was due to a raised resting concentration of intracellular calcium and hence a reduction in driving force, in the YAC mouse. The raised calcium concentration could indeed be due to enhanced NMDA receptor activity.

No changes in paired-pulse facilitation were observed in either mouse at any age tested. There was evidence of presynaptic alterations, however, as post-tetanic potentiation was reduced. It would appear, therefore, that presynaptic terminals react normally to a single stimulus, but are unable to sustain trains of repetitive transmitter release.

The authors assign some of their observations to altered NMDA receptor function (Hodgson *et al.*, 1999), however the caveat that Mg^{++}-free ACSF was used throughout is not considered.

1.4.2.3 R6/2 mouse

The third and perhaps the most comprehensive study to date was carried out within the hippocampus of the R6/2 mouse (Murphy *et al.*, 1998; 2000). Some basal properties and plastic changes were reported as abnormal, while others appeared normal. Within the CA1 region there was no age dependency observed for any of the reported changes below and therefore results from mice aged 5-18 weeks were pooled.

Resting membrane potential, membrane resistance and action potential threshold were found to be normal, while the amplitude and duration of the action potential were found to be reduced. This was reflected at the field level, where greater stimulation intensity was required to evoke a pop spike, although input-output relationships were found to be normal for both the EPSP and pop-spike field responses.

Long-term synaptic changes were reported as abnormal. LTP of the EPSP recorded from the CA1 field was impaired, while depotentiation was enhanced. The impairment of CA1 EPSP LTP was not age-dependent, with mice from five to 18 weeks displaying a similar deficit. In contrast LTP within the dentate gyrus *was* age-dependent with a greater impairment at >12 weeks than at 4-7 weeks.

PTP was found to be smaller in transgenics than in littermate controls, a difference ameliorated by the application of D-AP5, suggesting a modified NMDA receptor function rather than impaired transmitter release. Pharmacologically isolated NMDA-mediated EPSPs were, however, identical to littermate controls, suggesting a normal NMDA receptor function.

Interestingly, CA1 synapses were shown to support homosynaptic and reversible LTD, a phenomenon normally down-regulated by this age (Dudek & Bear, 1993; Milner *et al.*, 2004b). Furthermore, the induction of LTD was found to be D-AP5-sensitive (see figure 1.4.1).

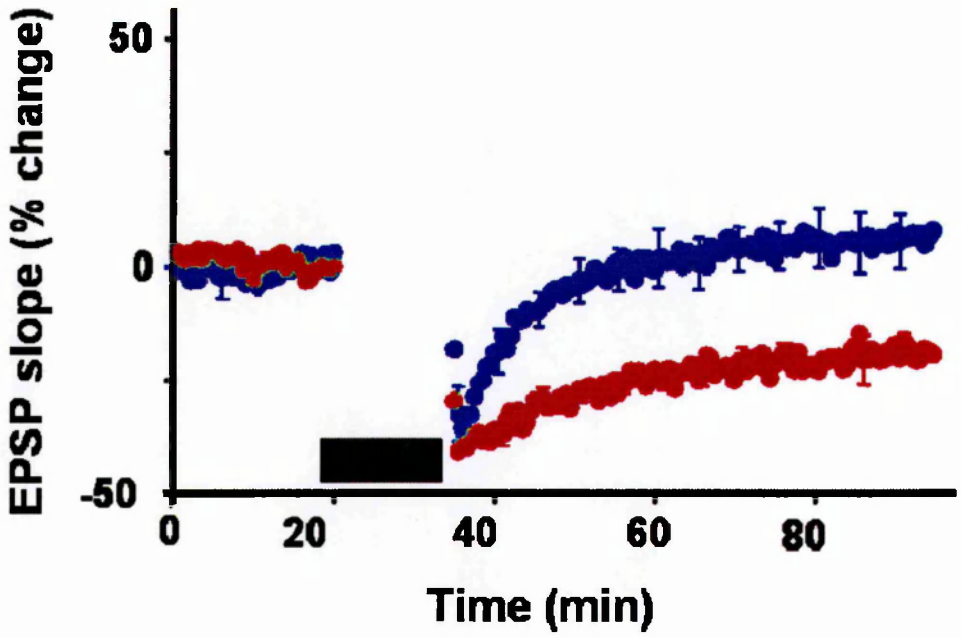


Figure 1.4.1: LTD in the R6/2 hippocampus. Low-frequency stimulation (solid bar) induces homosynaptic LTD at R6/2 (●) CA1 synapses, but not wild-type littermates (●). (Adapted from Murphy *et al.*, 2000.)

The development of inclusions within the hippocampus was also studied. Aggregate formation was developmental, with aggregates detectible in the CA1 field at 3 weeks, dentate area at 7 weeks and granule cells at 10 weeks. As the appearance of inclusions coincided with the development of both cognitive and synaptic impairments, the authors propose that inclusion formation may underlie the development of aberrant synaptic plasticity observed in these mice. This proposal is a very attractive one, suggesting that as mutant huntingtin builds up within the nucleus and aggregates, so the synaptic and cognitive phenotypes become apparent. In the reversible mouse model (Yamamoto *et al.*, 2000), switching off the transgene led not only to a clearance of aggregates but also a reversal of the behavioural (clasping) phenotype. This would suggest that aggregates are an important factor in disease progression. Conversely, there is a temporal difference between the rate of aggregate clearance and the reversal of the behavioural phenotype. Striatal cells, prepared from the double transgenic reversible mouse, showed aggregate formation within 2 days of gene expression and reached a plateau at 6 days. Following dox treatment to turn off gene expression, aggregates showed a decline within 3 days and were totally absent by 5 days of treatment (Martin-Aparicio *et al.*, 2001). Given this rapid turnover of aggregates *in vitro*, the temporal relationship between aggregate clearance and the behavioural phenotype was restudied. Aggregates were found to be totally cleared within 3 weeks of dox treatment. At 4 weeks, mice were still found to be deficient on a rotarod test and it was not until 8 weeks of treatment that transgenic mice were indistinguishable from age-matched controls (Martin-Aparicio *et al.*, 2001). While these results demonstrate that continued expression of mutant huntingtin is required for the maintenance of a phenotype, the disparity in aggregate

clearance and motor phenotype suggests that aggregates may not be an important factor in symptom development.

1.4.2.4 R6/1 mouse

The R6/2 mouse study of hippocampal synaptic plasticity (Murphy *et al.*, 2000) suggested there to be a progressive onset of an aberrant phenotype. This was particularly evident in the dentate area, while the CA1 field showed abnormal plasticity from an early age. The general deficit may be a reflection of the R6/2 mouse which is a relatively aggressive model of HD. In contrast the R6/1 mouse line is a more progressive model, with key phenotypic developments (including cognitive, motor and behavioural phenotypes) showing a greater separation than in the R6/2 (figure 1.4.2). Given its more protracted phenotype, the R6/1 mouse lends itself particularly well to linear studies. A study of LTD in the CA1-field of the R6/1 mouse has been carried out in our lab and preliminary data have been presented in abstract form (Milner *et al.*, 2002b; 2003a; Milner *et al.*, 2004a). The study demonstrates a normal developmental down-regulation followed by a re-emergence of LTD, even prior to mice exhibiting a behavioural phenotype.

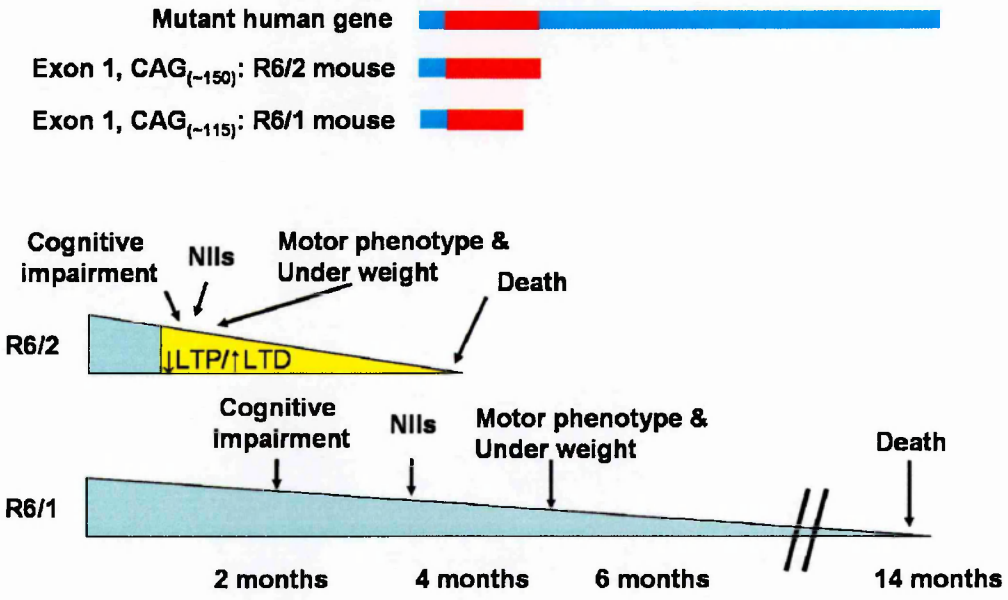


Figure 1.4.2: R6/2 versus R6/1 mouse model. *Top*, Representation of the HD mutation in humans (juvenile) showing an expanded CAG repeat in red. The truncated gene fragments inserted into the R6/2 and R6/1 mice are shown below. *Bottom*, timelines showing the advantages of the R6/1 mouse over the R6/2 mouse for linear studies of early onset phenotypes. In the R6/2 mouse the key phenotypic features manifest approximately simultaneously, whereas the R6/1 phenotype is more progressive, showing a gradual development closer to that seen the human syndrome.

1.4.2.5 Other models

Synaptic plasticity has also been briefly investigated in the reversible mouse model of HD (Yamamoto *et al.*, 2000). This model shows similar cognitive deficits to the R6/2 mouse which are correlated with a similar down regulation in LTP (see Bates & Murphy, 2002). As with the remarkable recovery and reversal of behavioural and pathological changes observed in this mouse following "gene-off", the electrophysiological and cognitive abnormalities also show a recovery.

The presynaptic SNARE-associated protein complexin II is down regulated in R6/2 mice (Morton & Edwardson, 2001). A complexin II knock-out mouse has been produced (Takahashi *et al.*, 1999). These mice show no abnormal behaviour and have normal basal synaptic transmission (input-output relationships and PPF). LTP recorded in both the CA1 field and the CA3 field showed impaired LTP compared to wild types, a finding very similar to that in the R6/2 (Murphy *et al.*, 2000).

1.4.2.6 Summary of synaptic plasticity in HD mouse models

A summary of the mouse models of HD can be found in table 1.4.1, which lists their key phenotypic features and the electrophysiological data. A consistent finding from the mouse models of HD is that synaptic plasticity is abnormal, even before the onset of a motor phenotype. Although the findings of Hodgson and colleagues are hard to directly compare with the other two reports because of the absence of magnesium in the ACSF, all three show that long-term potentiation is impaired compared to wild-type littermates. This finding is possibly mirrored in the striatum, where the

incidence of LTP may be increased, suggesting that a global deficit in LTP is not the case.

While the authors of the YAC and knock-in studies suggest a presynaptic modification may underlie abnormalities in plasticity, the R6/2 data would suggest a post-synaptic deficit involving downstream events of the NMDA receptor. The fact that the synaptic phenotype of the R6/2 and complexin II knock-out mice are similar (Takahashi *et al.*, 1999; Murphy *et al.*, 2000) would suggest, however, that a presynaptic mechanism underlies the deficits observed in the R6/2 mouse. A complexin II deficit (Morton & Edwardson, 2001) could also account for the impairments observed in sustained neurotransmitter release at high frequency. A lack of inhibitory complexin II in HD could lead to a disinhibition of transmitter release resulting in rapid synapse depletion during periods of high-frequency activity (as described by Usdin *et al.* 1999).

	Mouse Model	Gross Phenotype								Huntingtin Localisation				Striatal Protein Neurochemistry				Corticostriatal Transmission & striatal MSN cell properties									Hippocampal Plasticity					
		$(CAG)_n$	Prot. Level (vs Hdh)	Motor Phenotype	Cog/Behav/Activity	Wight Loss	Brain Weight Loss	Neurodegeneration	Astrogliosis	Nuclear Stain	Nuc. Microagg.	Nuc. Inclusions	Neuropil Agg.	Enkephalin	Substance P	DAR	NMDA (NR1)	RMP	R_{in}	Tau	AP	EPSP	I/O	PPF	NMDAR Current	Age Dependent	I/O	PPF	PTP	LTP	NMDAR	Age-dependent
Truncated	R6/2	150	↑	✓	✓	✓	✓	✓	×	nr	nr	✓	✓	↓	↔	↓	↔[a]	↓	↑	↓	↓	↓	↓	↓	↑	✓	↔	↔	↓	↓	↔	✓
	HD100	100	↓	✓	✓	nr	nr	✓	×	✓	nr	✓	nr	↓	↔	↓	nr	↔	↓	nr	↔	↓	↓	nr	↑	✓	nr	nr	nr	nr	nr	nr
	N171-82Q	82	↓	✓	nr	✓	✓	✓[c]	×	✓	nr	✓	✓	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr
	HD94Rev	94	nr	✓	nr	×	✓	×	✓	✓	nr	✓	✓	nr[m]	nr	↓	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr
Full length	YAC46	46	↓	×	×	×	×	×	×	×	×	×	×	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	↔	↓[f]	↓[f]	↔[f]	✓
	YAC72	72	↓[d]	✓	✓	✓[d]	✓[d]	✓	nr	✓	✓	✓[d]	nr	↓	↔	↓	nr	↔[e]	↔[e]	↔[e]	nr	nr	nr	nr	↑	nr	nr	↔	↓[f]	↓[f]	↔[f]	✓
	HD48	48	↑	✓	✓	✓	nr	✓	✓	✓	nr	✓	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr
	HD89	89	↑	✓	✓	✓	nr	✓	✓	✓	nr	✓	nr	nr	nr	↓	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr
Knock-in	CAG71	71	↔	×	×	×	×	×	×	×	×	×	×	↔[g]	↔	nr	nr	↓[b]	↔	nr	↔	nr	nr	nr	↑	nr	nr	nr	nr	nr	nr	nr	nr
	CAG94	94	↔	✓	✓	×	↔[h]	×	nr	×	✓	✓	×	nr	nr	nr	nr	↓[b]	↔	nr	↔	nr	nr	nr	↑	nr	nr	nr	nr	nr	nr	nr	nr
	CAG140	140	↔	✓	✓	×	↔[h]	nr	nr	✓	✓	✓	✓	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr
	Hdh6/Q72	72	↓	×	✓	×	×	✓	×	✓	nr	✓	✓	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	↔	↓	↓	↓	↔	nr
	Hdh4/Q80	80	↓	×	✓	×	×	✓	×	✓	nr	✓	✓	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	↔	↓	↓	↓	↔	nr
	CHL1	80	↔	✓	nr	✓	×	×	×	×	nr	×	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr
	CHL2	150	↔	✓	nr	✓	×	×	✓	✓	nr	✓	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr

Table 1.4.1: Summary of the mouse models of HD. ↑= increase; ↓= decrease; ↔= no change; × = no; ✓ = yes; nr = not reported; [a] = altered subtype ratio NR1 increased, NR2A/B decreased; [b]= Subpopulation only; [c]= possible degeneration (note added in proof); [d]= high expresser (twice *Hdh*) only (lower expresser (1/3 *Hdh*) no change); [e]= trend suggests same alterations as in R6/2, however numbers too low; [f] = experiments carried out in 0 Mg^{++}; [g]= mRNA reduced; [h]= reduction in striatal volume. Produced in collaboration with Austen J. Milner; See main text for references.

1.4.3 Summary

Evidence obtained from electrophysiological studies within the hippocampus of mouse models of HD demonstrates that synaptic plasticity is abnormal within these mice. There are, however, no data on electrophysiological studies of long-term synaptic plasticity within the cerebral cortex. The cerebrum is a region that undergoes severe neurodegeneration by the later stages of HD. Prior to the onset of the motor and psychiatric conditions that are associated with cell death, there are early cognitive deficits that correlate with loss of neurochemical markers. In particular, impairments in recognition memory are discernable before other cognitive deficits. Evidence suggests that aspects of recognition memory are encoded within a region of the telepencephalon known as the perirhinal cortex. The cellular mechanism by which recognition memory is encoded within this region is thought to be that of long-term depression. Given the aberrant up-regulation of LTD in the hippocampus of R6/2 mice, it is reasonable to suggest that LTD may be abnormal in regions, such as the perirhinal cortex, where LTD is a normal form of synaptic plasticity.

1.5 The perirhinal cortex

The perirhinal cortex is located within the medial temporal lobe (for review see Burwell *et al.*, 1995). More specifically it is located at the posterior region of the rhinal sulcus and encompasses areas 35 and 36 as illustrated by Korbinian Brodmann in the human brain (Brodmann, 1909, figure 1.5.1). The perirhinal cortex has been demonstrated to be involved in various cognitive tasks (for review see Suzuki, 1996; Murray & Bussey, 1999) including stimulus-stimulus associations (usually evaluated by cue associations: "If cue A then respond X but not Y, if cue B then respond Y not X", Murray *et al.*, 1993; Buckley & Gaffan, 1998; Bussey *et al.*, 2003), fear conditioning (the acquirement of a neutral stimulus to evoke a strong emotional response following temporal pairing with an aversive stimulus, Corodimas & LeDoux, 1995) and also a more general role in visual processing, for example the retention of previously learned object discriminations are lost following lesion of the perirhinal cortex (Gaffan & Murray, 1992; Buckley & Gaffan, 1997; Thornton *et al.*, 1997). There is also strong evidence that the key role of the perirhinal cortex is that of discrimination between novel and familiar cues (Brown & Xiang, 1998; Brown & Aggleton, 2001). This type of discrimination is known as recognition memory which will be discussed in section 1.5.2.

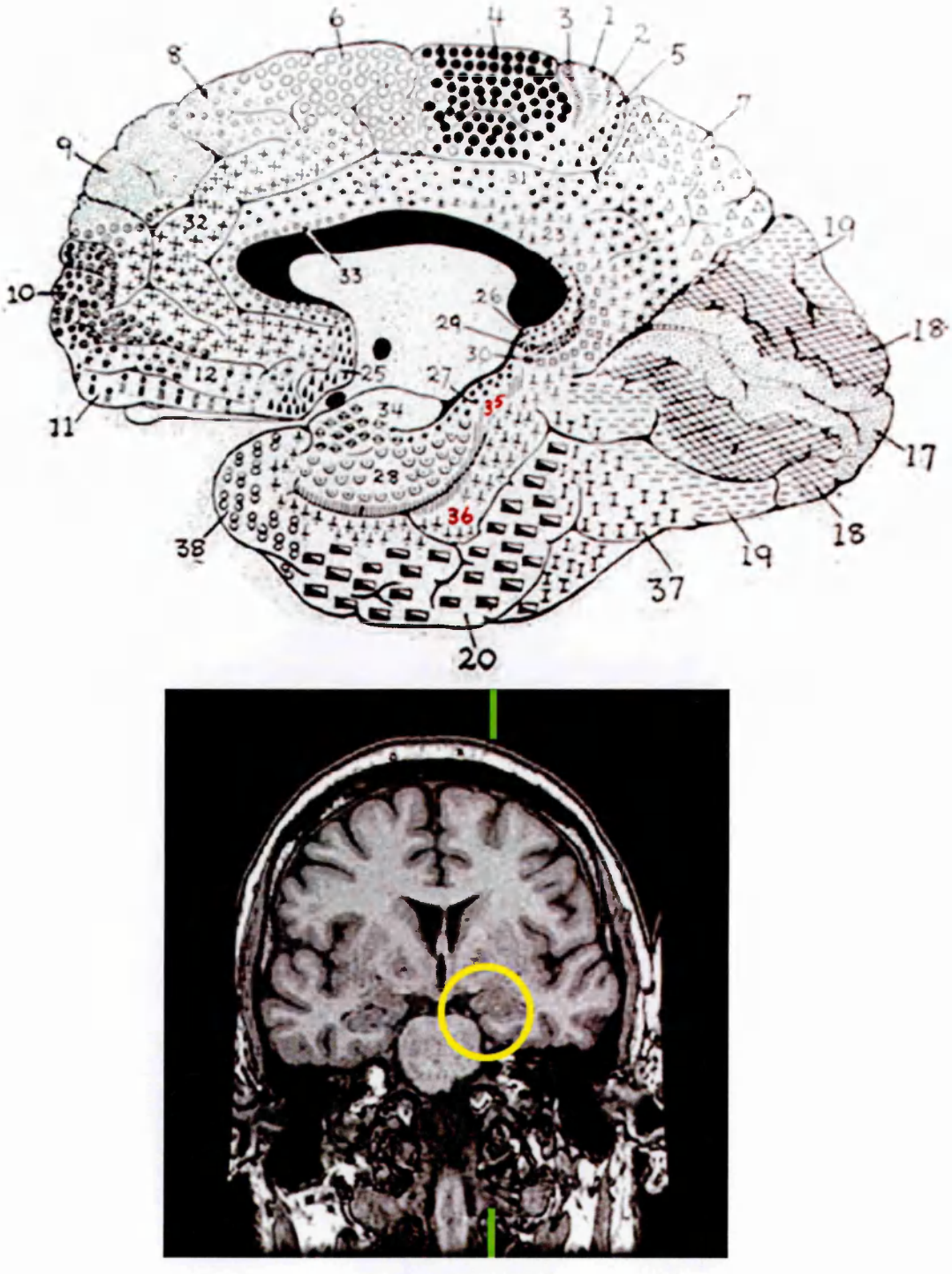


Figure 1.5.1: Location of the human perirhinal cortex. *Top,* One of Brodmann's original illustrations showing areas 35 and 36 (red numbers) which make up the perirhinal cortex. (Adapted from Brodmann, 1909.) *Bottom*, An MRI scan of the human head. The location of the perirhinal cortex is highlighted by the yellow circle. The green line indicates the approximate plane of the Brodmann figure above (Adapted from Johnson & Becker, 1995-1999.)

1.5.1 Neuroanatomy

The neuroanatomy of the perirhinal cortex has been described in several species including monkey (Suzuki & Amaral, 1994a, b), cat (Witter & Groenewegen, 1986), dog (Woznicka & Kosmal, 2003) and rat (Burwell *et al.*, 1995). The same general structure is common in all species, with the perirhinal cortex located in Brodmann areas 35 and 36 along the full extent of the rhinal sulcus and is bordered by entorhinal and temporal association cortices. The rodent perirhinal cortex is shown in figure 1.5.2

In rat (and other species) area 35 and area 36 have distinct architectures (see (Burwell & Amaral, 1998; Burwell, 2001). While area 36 has a granular layer IV, area 35 is considered agranular, having only few granule cells dispersed into the pyramidal cells of the inner region of layer III and outer region of layer V. In both areas layer I is (almost) cell free, containing collateral inputs from adjacent regions. Layers II/III are densely packed with medium-sized pyramidal cells, while the pyramidal layer V contains widely spaced, large pyramidals. Layer VI can be separated into superficial and deep regions; the superficial region consists of polygonal cells; the deep region contains elongated, flat cells. Below layer VI is found the corpus callosum (white matter) and then the hippocampal formation. The interconnectivity of the cellular layers is represented in figure 1.5.2.

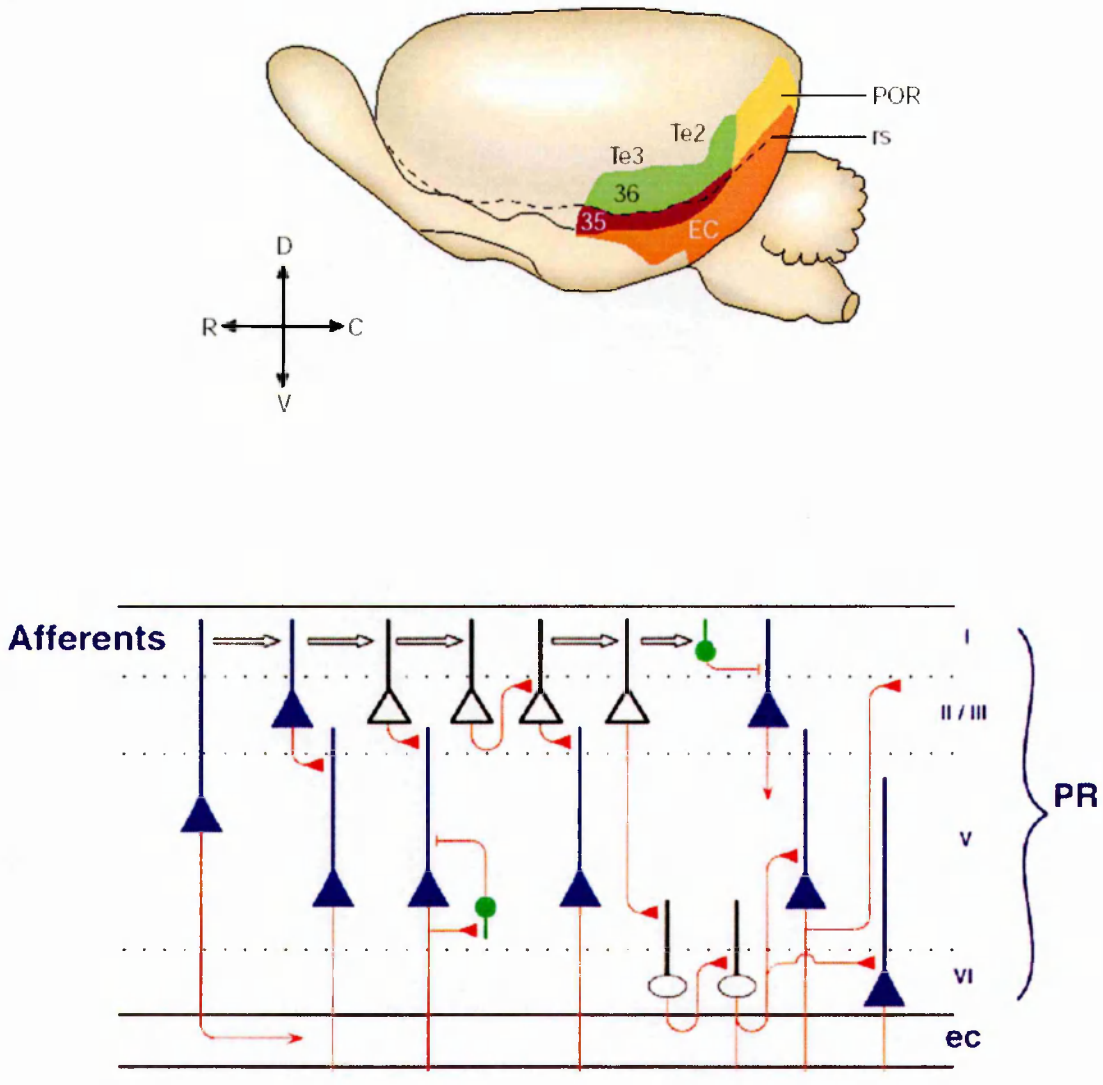


Figure 1.5.2: The rodent perirhinal cortex. *Top*, Lateral view of the rodent brain showing the location of areas 35 and 36, the perirhinal cortex, which sits along the extent of the rhinal sulcus (rs). Bordering this region are the entorhinal cortex (EC), temporal cortex (Te) and postrhinal cortex (POR). (Adapted from Brown & Aggleton, 2001.) *Bottom*, Schematic of the cytoarchitecture of the perirhinal cortex. Afferents synapse with layer II/III pyramidal cells in the surface layer. Pyramidal cells project to other pyramidals in both layer II and in the deeper layers V and VI. Interspersed in layer V are granule cells which provide an inhibitory input. The axonal output of the perirhinal cortex (red lines) projects to other regions of the brain, see figure 1.5.3. (Adapted from Faulkner & Brown, 1999.)

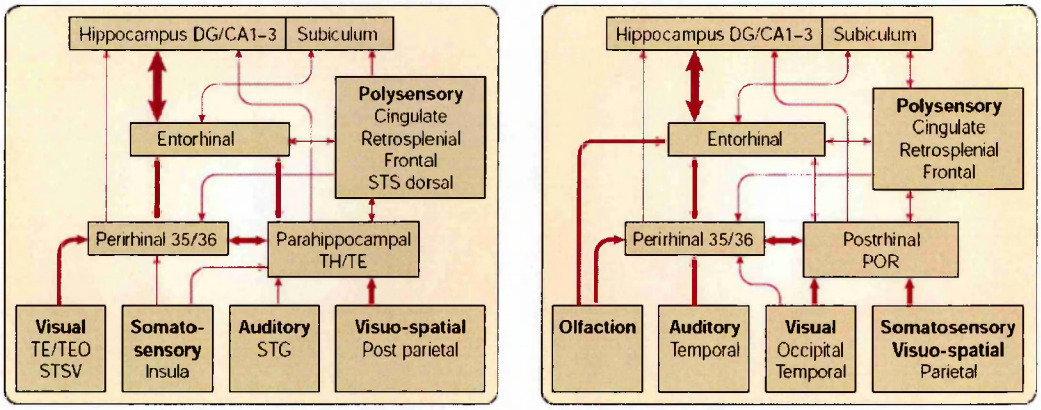


Figure 1.5.3: The connectivity of the perirhinal cortex with other cortical regions. Connectional diagrams showing the parallel routes by which sensory information reaches the perirhinal cortex and adjacent regions. The thickness of the arrows indicates the size of the projection (DG, dentate gyrus; EC, entorhinal cortex; POR, postrhinal cortex; STG, superior temporal gyrus; STS, superior temporal sulcus; TE, temporal cortex). (Adapted from Brown & Aggleton, 2001.)

1.5.1.1 Glutamatergic pathways

There are three major connections between the perirhinal cortex and other brain regions (see Suzuki, 1996): 1) Reciprocal projections with the hippocampus and the entorhinal cortex; 2) Inputs from both unimodal and polymodal association cortices; 3) Interconnections with the amygdaloid complex. The main connections of the perirhinal cortex are summarised in figure 1.5.3.

The above connections are not uniformly distributed between areas 35 and 36 (Burwell & Amaral, 1998). Temporal cortex provides the source of afferentation for 50% of all inputs into area 36, but only 10% of area 35. In contrast, area 35 receives 22% of its inputs from entorhinal cortex and 12 % from temporal cortex. Projections from layer V cells terminate on layer V perirhinal cells. Inputs to layer III cells originate in layer III of adjacent cortex. Likewise area 35 receives 25% of its cortical input from the piriform cortex, which provides only 6% of the input to area 36. These afferents terminate in the superficial layers and are represented in figure 1.5.2.

1.5.1.2 Dopaminergic pathways

An input that is commonly overlooked when considering the perirhinal cortex is that of dopaminergic connections from the mesolimbic system. The mesolimbic system contains dopaminergic neurones within areas A8 and A9 (substantia nigra) and A10 (ventral tegmental area, VTA) of Dahlström and Fuxe (1964). These pathways are thought to be involved in reward and pleasure and long-lasting enhancements in these pathways are thought to underlie opiate addiction (see Thomas & Malenka,

2003). There is good evidence that the perirhinal cortex receives a substantial afferentation from the VTA neurones, but not particularly from the substantia nigra.

It was realised in the early 1970s that cortical innervation by dopaminergic neurones was evident (Thierry *et al.*, 1973a; 1973b; Berger *et al.*, 1974; Lindvall *et al.*, 1974). Initial studies highlighting a dopaminergic input into the perirhinal cortex were carried by dopamine uptake experiments, visualised by fluorescence following lesions of the noradrenergic neurones. This technique showed that all regions of cortex have dopaminergic fibres, but the region immediately dorsal to the rhinal sulcus in rat receives a high proportion of the overall cortical dopaminergic innervations. Other cortical areas particularly rich in innervation were the prefrontal and frontal cortices (Berger *et al.*, 1976).

A more refined study was carried out by Simon and colleagues using anterograde tracing. Tritiated leucine was injected into VTA neurones and found to project through the medial forebrain bundle to the amygdala and cortical regions (Simon *et al.*, 1979). Although the perirhinal cortex was not specifically analysed, there is clear labelling within the region, demonstrating a direct path from VTA to perirhinal cortex.

The first quantitative study of dopaminergic afferentation of the perirhinal cortex was carried out again using tritiated dopamine. Two groups of rats were studied, one normal and one that received 6-hydroxydopamine lesions to the VTA. Following lesioning, slices were taken from rats and incubated in a chamber containing a monoamine oxidase inhibitor and desipramine (a drug that prevents monoamine uptake into noradrenergic and 5-HT neurones), exposed to the tritiated dopamine and then fixed for histochemical examination and measured per cubic millimetre of tissue. Labelled varicosities were found to be particularly high in the

cingulate and prefrontal cortices; the perirhinal, entorhinal and piriform cortices received an intermediate degree of innervation; and other cortical regions, including visual and parietal cortices, showed only a low level of labelling (Descarries *et al.*, 1987). The rats that received lesions showed almost no cortical labelling. The laminar densities were assed to a degree and found to be higher in layers III-VI (as assessed as a whole) than in layers I/II.

Consistent with the dopaminergic afferentation of the perirhinal cortex a number of autoradiographic studies have demonstrated the presence of both D_1 and D_2 dopamine receptors. In these experiments tritiated receptor-specific ligands were applied to sections of perirhinal cortex from a range of species to identify the distribution of receptors. The first of these reports showed that, in rats, cats and macaque monkeys, there is laminar distribution of both receptor subclasses (Richfield *et al.*, 1989). D_1 receptors were present at an order of magnitude higher than D_2 receptors in all layers of cortex from all species. Furthermore layers I/II had the most intense labelling of both receptors, closely followed by layers V/VI; layers III/IV were always lower than the other two bands.

Another autoradiographical study was carried out examining only D_2 dopamine receptors in rat, cat and human temporal cortex. Similar results to the aforementioned article was found for the human perirhinal cortex (Goldsmith & Joyce, 1994, 1996). The rat and cat perirhinal cortex, however, showed a different trilaminar distribution (although it should be noted that the representative autoradiograph is far from convincing). There was intense emission from layers I and III, while the deeper layers were totally devoid of labelling, as were the entorhinal and temporal cortices (Goldsmith & Joyce, 1994).

Also consistent with the human studies is the distribution of the dopamine transporter in the human perirhinal cortex. Dopamine transporter-containing fibres were found in all layers and staining was particularly intense in the surface and deep layers (Ciliax *et al.*, 1999), consistent with both the innervation and dopamine receptor distribution.

In light of the two contrasting autoradiographical reports, it is apparent that the distribution of D_2 (and, with the lack of replication, D_1) is questionable within rodents and should, therefore, be addressed.

It appears, therefore, that inputs from multiple regions converge at the perirhinal cortex. This is consistent with the different cognitive functions in which the region has been implicated i.e. the declarative aspects can be associated with the hippocampal connections; emotional memories with the amygdala; and associative properties with other regions of cortex. The dopaminergic input from mesolimbic regions may provide a reward aspect to cognitive processes encoded within the perirhinal cortex.

1.5.2 Recognition memory

Several lines of evidence implicate the perirhinal cortex as having a crucial role in recognition memory (see Brown & Xiang, 1998; Brown & Aggleton, 2001). Recognition memory requires the ability to discriminate between a novel or familiar cue and judgement concerning prior occurrences, for example recency or context. Various studies have implemented temporal lobe structures as being important in

recognition memory. Amnesic patients that have suffered sustained ventromedial temporal cortical damage show impairments in recognition memory (Aggleton & Shaw, 1996). Similarly, bilateral ablation of the hippocampal formation, the amygdala and adjacent cortex, including entorhinal and perirhinal cortices, in monkeys produces severe memory impairments (Mishkin, 1978; Zola-Morgan *et al.*, 1982; Murray & Mishkin, 1984).

In an attempt to dissect the exact roles of the amygdala and hippocampus in recognition memory, Murray and Mishkin (1986) removed the rhinal cortices in conjunction with either the amygdala or the hippocampus. Monkeys that underwent hippocampectomy were found to be far less impaired than those that underwent the amygdalectomy. Furthermore, the amygdalectomised group were equally as impaired as those that had undergone complete removal. Similarly, in humans, amnesic patients with damage restricted to the hippocampus perform well in recognition tests (Murray & Bussey, 1999).

The role of the amygdala was later called into question when it was found that bilateral lesions of the amygdala, which spared the overlying cortex, did not exacerbate the memory loss arising from hippocampectomy alone (Zola-Morgan *et al.*, 1989). This suggests that neither the hippocampus, nor the amygdala are the location of recognition memory encoding, but rather the overlaying cortex. Further evidence for the exclusion of the amygdala in a role in recognition memory came following new architechtonic and connectional studies in 1987, when it was realised that the perirhinal cortex extends far more rostral than previously considered (Amaral *et al.*, 1987; Insausti *et al.*, 1987). Following this new definition of the perirhinal cortex the results of the 1986 Murray and Mishkin paper were reassessed and it was found that the cortical damage caused by the amygdalectomy was concentrated far

more on the perirhinal region than first appreciated. Experiments were carried out to separate the contributions provided by the entorhinal and perirhinal cortices and it was found that entorhinal (only) ablation in monkeys led to only mild impairments of recognition, while perirhinal (only) ablations led to severe impairments (Meunier *et al.*, 1993). This perirhinal cortical specific impairment was almost as severe as that seen with the total parahippocampal ablations (cross-laboratory comparisons). Furthermore, the impairments following perirhinal ablations applies to both visual and tactual recognition (Suzuki *et al.*, 1993).

It appears, therefore, that the ability to discriminate between novel or familiar cues is encoded within the perirhinal cortex, while contextual elements of recognition memory are encoded in perirhinal cortex-associated brain regions. Pharmacological studies have implicated a range of neurotransmitter systems in recognition memory.

Glutamatergic transmission is important in object recognition. Blockade of AMPA/KA receptors by CNQX prevents the acquisition of object recognition memories in rats (Barker *et al.*, 2003). Reports of NMDA receptor involvement in recognition memory varies. MK801 blockade of NMDA receptors does not affect novel object exploration, but does affect contextual (spatial) aspects of recognition memory (Bevins & Bardo, 1999). In contrast, application of high (but not low) doses of D-AP5 does affect the performance of rats in recognition tasks (Abe *et al.*, 2004). Metabotropic glutamate receptors appear to be involved in longer-term recognition memory (more than 24 hours) but not short-term memory (20 minutes), as demonstrated by the broad-spectrum antagonist LY341495(Barker *et al.*, 2003). When group-specific antagonists were used, it was found that neither mGlu5, group II nor group III-specific antagonists significantly impaired object recognition at 24

hours. In contrast, co-application of the mGlu5 and group II antagonists impaired the rats performance (Barker *et al.*, 2003/ personal communication).

Dopaminergic activity is important for recognition memory. Blockade of either D_1 dopamine receptors with SCH23390 (Besheer *et al.*, 1999) or D_2 receptor antagonism with raclopride (Woolley *et al.*, 2003) reduces the time spent by rats exploring novel objects in an open field arena test. A role for dopaminergic pathways in recognition memory is also provided by methamphetamine-induced monoamine toxicity, which also impairs a rats ability to perform on these tasks (Bisagno *et al.*, 2002; Belcher *et al.*, 2003).

Inhibition of acetylcholine activity at cortical and hippocampal muscarinic receptors by scopolamine impairs object recognition in both young (3-6 months) and old (20-24 months) rats (Vannucchi *et al.*, 1997). The cholinergic-induced deficit is dose-dependent and has been shown to be reversed by an antagonist of the 5-HT_6 receptor (Woolley *et al.*, 2003) or 5-HT_1 receptor (Pitsikas *et al.*, 2003). Similarly, the 5-HT_6 antagonist reverses impairment in recognition memory in rat induced by the D_2 dopamine receptor antagonist raclopride (Woolley *et al.*, 2003).

1.5.3. Neural correlates of recognition memory

Several electrophysiological studies have identified a potential neural correlate for recognition memory. Recordings from neurones in the monkey perirhinal cortex show that some cells fire fewer action potentials upon the second and subsequent presentation of stimuli than on the first (figure 1.5.3, Brown *et al.*, 1987; Fahy *et al.*, 1993; Xiang & Brown, 1998). Approximately 25% of all recorded neurones show decrements in firing rate upon repeated cue presentation (Miller *et al.*, 1993; Xiang & Brown, 1998). Of these cells, 50% show such reductions for more than 24 hrs after the first presentation (Xiang & Brown, 1998). The change in firing rate occurs within 90 ms of the stimulus presentation. This rapid response is too fast for the involvement of feedback from other brain regions (Brown & Bashir, 2002) meaning that familiarity encoding is inherent to the perirhinal cortex. Importantly, reductions in neuronal firing rate are seen independently of reward or even prior training on recognition tasks (Fahy *et al.*, 1993; Brown & Xiang, 1998; Brown & Bashir, 2002) and therefore the decrements in firing rate are intrinsic to familiarity. The system has a high capacity, which is demonstrated by the observation that such reductions in neuronal firing are seen despite the animal having seen many hundreds of items (Xiang & Brown, 1998). Similar reductions have also been reported in the anaesthetised (Zhu & Brown, 1995) and awake (Zhu *et al.*, 1995) rat perirhinal cortex, indicating that this mechanism is common to other species.

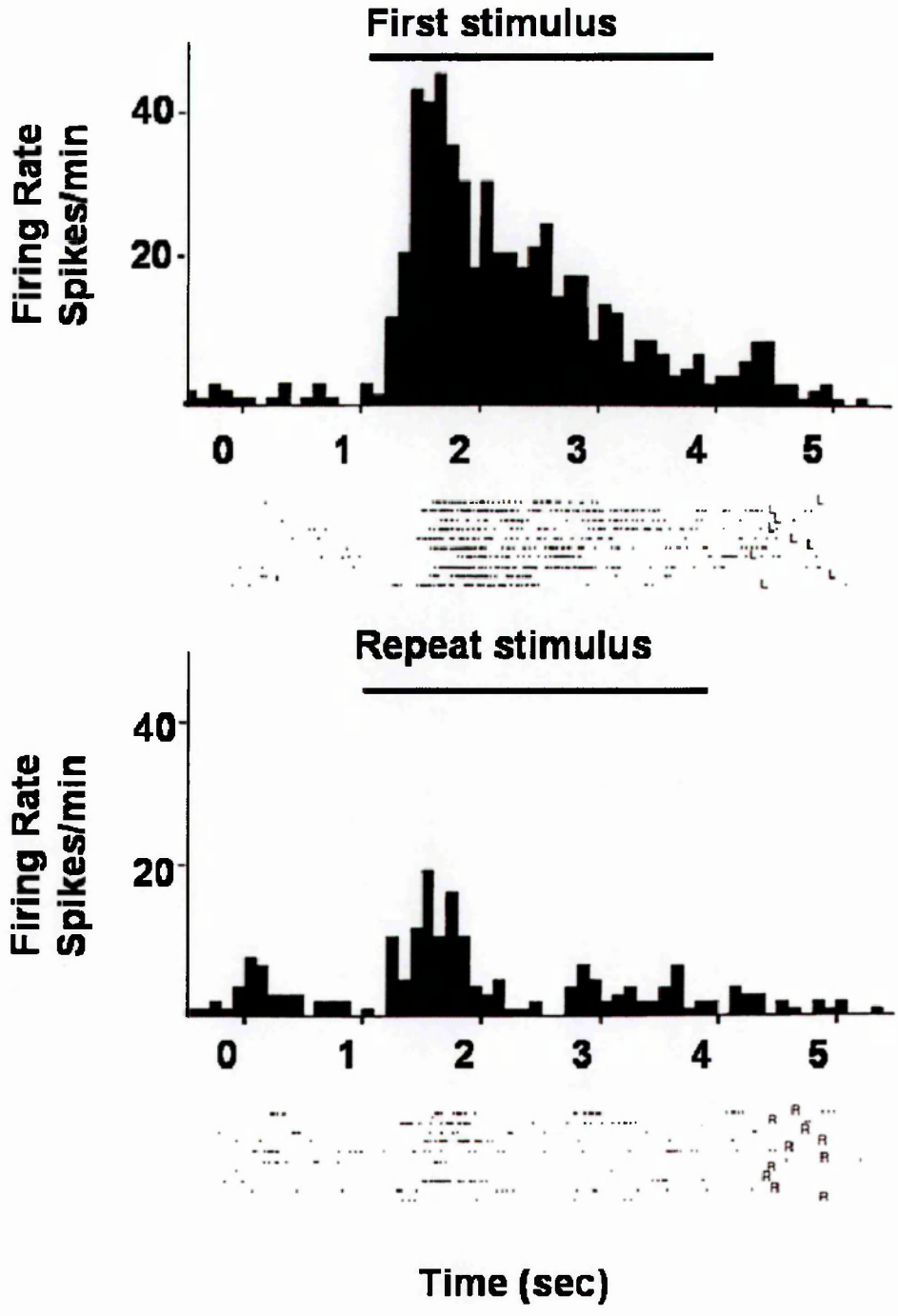


Figure 1.5.4: An example of decremental repetition-related neuronal response. Note the much weaker response of perirhinal neurones to the repeat stimulus (lower histogram and raster plot) compared to the first stimulus (upper histogram and raster plot). Responses were recorded from monkey perirhinal cortical neurones during a serial recognition memory task. 10 different pictures were presented to the monkey for three seconds (bar) and the occurrences of action potentials in each trial are represented as dots in each row of the raster. Variable numbers of trials using other pictures intervened between the first and second presentation of each stimulus used in the first trial. The order of the ten raster plots is the same for both the first and repeat traces. (Adapted from Brown & Xiang, 1998.)

1.5.4. Plasticity in the perirhinal cortex

It is possible that changes in synaptic transmission at perirhinal cortical synapses may underlie the changes in neuronal firing rate observed during familiarisation. Plastic changes in synaptic transmission have been reported in the perirhinal cortex. Both long-term potentiation (Bilkey, 1996; Ziakopoulos *et al.*, 1999) and long-term depression (McCaffery *et al.*, 1999; Cho *et al.*, 2000) can be expressed in acute slices taken from rat perirhinal cortex. It is likely that long-term depression is the underlying mechanism by which a decremental neuronal firing rate is mediated.

Short-term changes

Application of paired-pulse stimuli to perirhinal cortical synapses results in depression of the second response (Paired-pulse depression; PPD; figure 1.5.5). This is in contrast to the response obtained in the hippocampus where PPF is observed. In the hippocampal system, PPF is known to be a presynaptic phenomenon involving calcium preloading of the presynaptic terminal to affect greater transmitter release on the second stimulus. PPD results form modulation of this mechanism by the expression of presynaptic autoreceptors, commonly metabotropic glutamate receptors (Scanziani *et al.*, 1997; Vogt & Nicoll, 1999).

Within the rat perirhinal cortex PPD is observed between stimulus intervals of 25 and 2000 ms, with a maximal depression at 200 ms. The paired-pulse profile closely follows the temporal profile of $GABA_B$ mediated inhibitory postsynaptic potentials, suggesting that $GABA_B$ receptors may mediate the PPD observed.

Indeed, blockade of $GABA_B$ receptors alters the profiles to that of facilitation (Ziakopoulos *et al.*, 2000). PPD was not affected by CPPG (group II/III mGlu receptor antagonist at the concentration used) or the adenosine A1 receptor antagonist DPCPX (Ziakopoulos *et al.*, 2000 235). PPD in the rat perirhinal cortex was found to be heterosynaptic, such that either temporal followed by entorhinal stimulation, or *vice versa*, resulted in PPD.

Long-term depression

Long-term depression was first demonstrated in the perirhinal cortex by pharmacological activation of mGlu receptors using group-specific agonists (McCaffery *et al.*, 1999). The broad spectrum agonist of group I/II mGlu receptors, (1*S*, 3*R*)-1-aminocyclopentane-1,3,dicarboxilic acid (ACPD), bath applied to slices of rat perirhinal cortex, induced a robust depression in synaptic transmission that lasted for over 60 minutes following washout of the drug. A less consistent result was obtained with the group I mGlu receptor specific agonist (*R, S*)-3,5-dihydroxyphenylglycine (DHPG). Group II mGlu receptor agonism using (2*S*, 2'R, 3'R)-2-(2', 3'-Dicarboxycyclopropyl)glycine (DCG-IV) induced a significant and long-lasting depression. In contrast, activation of the group III subclass of mGlu receptors by the specific agonist (*S*)-2-amino-4-phosphonobutanoate (L-AP4) failed to induce a lasting depression in synaptic transmission following washout of the drug.

Initial attempts to induce an extracellularly recorded LTD resulted in depression that returned to approximately 10% of baseline within 30 minutes and had essentially returned to baseline one hour post conditioning (Ziakopoulos *et al.*, 1999,

figure 1.5.5). The maximal degree of depression obtained in the temporal pathway was significantly greater than in the entorhinal input. The maximal depression exhibited by the temporal, but not the entorhinal pathway, was reduced by application of D-AP5. Neither pathway was affected by the mGlu receptor antagonist MCPG (Ziakopoulos *et al.*, 1999). This would suggest that NMDA, but not mGlu receptors, are involved in this short-lasting depression.

In contrast, intracellularly recorded currents did show LTD in response to low-frequency stimulation (Cho *et al.*, 2000, figure 1.5.5). An interesting activity-dependent form of LTD was found to be supported at perirhinal synapses that is co-dependent on both NMDA and mGlu receptors. The co-dependency is a feature that is shared with only one other brain area: The amygdala (Wang & Gean, 1999). Interestingly mGlu receptors demonstrate a voltage-dependency for involvement in LTD induction. When perirhinal neurones were voltage clamped at –70 mV, LTD was found to be dependent on the co-activation of the NMDA receptor and both group I and group II mGlu receptors. When the cell was depolarised to –40 mV, however, the requirement of group II mGlu receptor activation was removed (Cho *et al.*, 2000). One plausible explanation of the voltage-dependent involvement of group II mGlu receptors is a synergistic interaction between group I and II mGlu receptors (Schoepp *et al.*, 1996; Mistry *et al.*, 1998) such that there is enhanced calcium release from intracellular stores when calcium influx though the NMDA channel is limited (i.e. at –70 mV). It appears, therefore, that the rat perirhinal cortex supports a novel form of LTD.

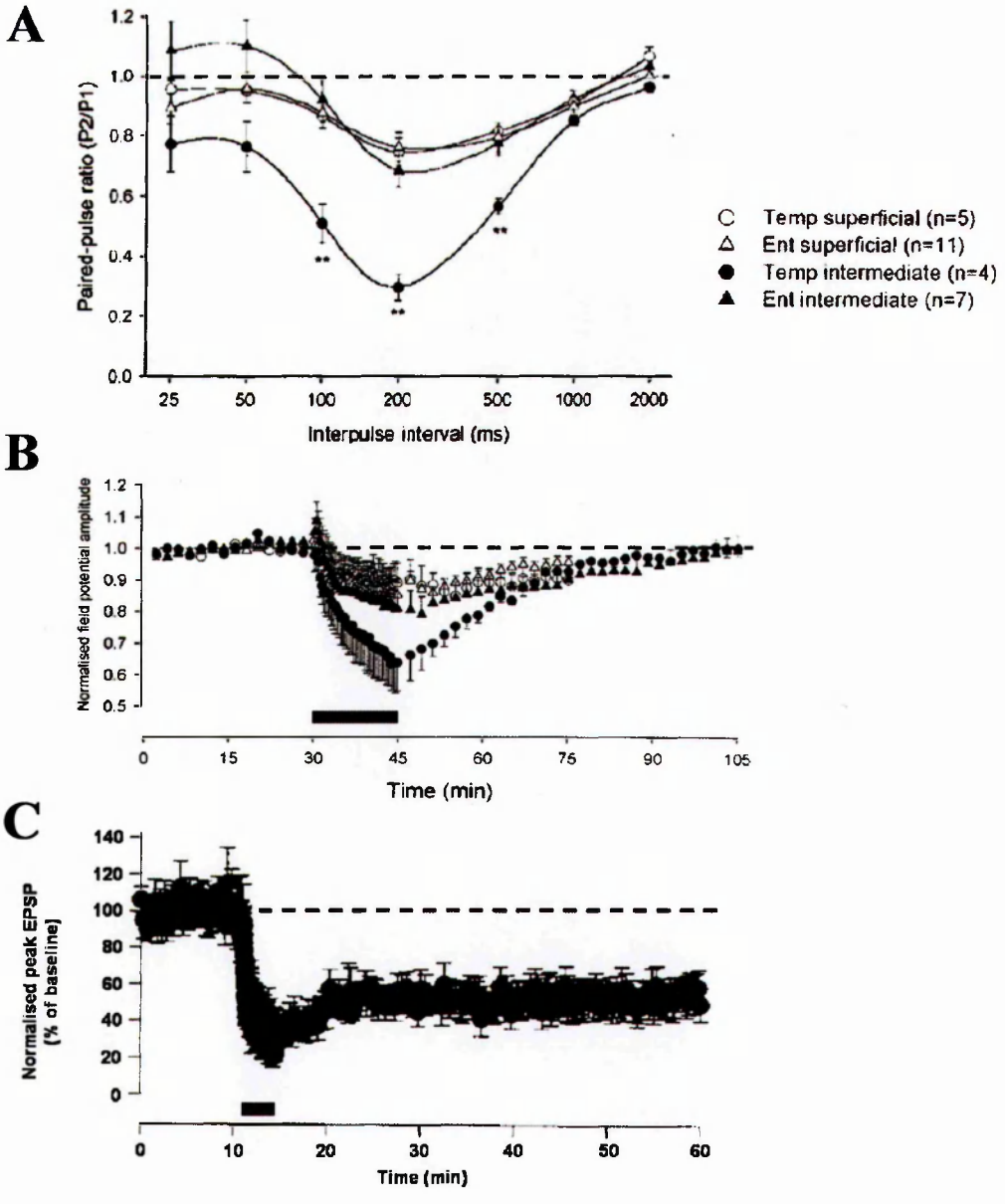


Figure 1.5.5: Synaptic plasticity in the rat perirhinal cortex. A, Paired-pulse depression resulting from paired stimuli applied to either the superficial or intermediate (layer I or layer III) afferents of the temporal and entorhinal pathways (logarithmic scale). No difference was observed between the entorhinal and temporal superficial pathways, while the intermediate temporal pathway showed a greater degree of depression (**= $P<0.005$). B, Synaptic depression recorded at the field level in the perirhinal cortex. While LFS (bar) led to a frequency-induced depression, responses returned to baseline within one hour. The degree of depression observed in the intermediate temporal pathway was greater than that observed in the other pathways. (A & B adapted from Ziakopoulos *et al.*, 1999.) C, Long-term depression recorded intracellularly. LFS induces a homosynaptic LTD of synaptic currents as recorded from the intermediate pathway (no difference between temporal and entorhinal). (Adapted from Cho *et al.*, 2000.)

Modulation of LTD

Other classes of receptor have been confirmed as playing a role in the induction of LTD at rat perirhinal cortical synapses. As discussed above, muscarinic acetylcholine receptors appear to be involved in recognition memory (see also Warburton *et al.*, 2003). A muscarinic receptor antagonist also prevents the decrease in neuronal firing observed in rats during exploration of novel objects and also blocks the induction of activity-dependent LTD (Warburton *et al.*, 2003). Conversely, the application of the cholinergic agonist, carbachol, induces LTD (Massey *et al.*, 2001). Agonist-induced LTD was prevented by pirenzepine (an antagonist of the M1 class of muscarinic acetylcholine receptor, but was not affected by the NMDA receptor antagonist D-AP5 (Massey *et al.*, 2001).

Despite the evidence for a substantial dopaminergic innervation of the perirhinal cortex and the demonstration that antagonists of dopamine receptors affect recognition memory, to my knowledge no electrophysiological investigations have been targeted towards the modulation of glutamatergic transmission and plasticity by dopamine receptors within this region.

1.5.5 Synaptic integration

A complementary mechanism to that of LTD of synaptic transmission has been suggested in which synaptic activity may give rise to altered firing patterns of perirhinal cortical neurones. Thomas Brown and co-workers have identified multiple cell types within the perirhinal cortex based both on morphology and their electrophysiological properties (Faulkner & Brown, 1999). The pyramidal cells of the perirhinal cortex can be split into three sub groups based on their firing patterns. Regular-spiking cells are characteristic of many glutamatergic cell-types within the brain, in that they fire action potentials near to the beginning of and throughout, a depolarising current step and show accommodation (figure 1.5.6). In contrast, late-spiking cells show a delayed onset firing of up to several seconds delay (figure 1.5.6). The third class, burst-spiking cells, show a stereotyped cluster of firing upon depolarisation, followed by a prolonged after hyperpolarisation.

Within layer II/III, 78% of pyramidal neurones studied by Faulkner and Brown (1999) were late-spiking cells and 22% regular-spiking cells. In layer V, 80% of pyramidal cells were regular-spiking neurones and the remaining 20% were burst-spiking neurones. Other cells types identified within the perirhinal cortex include fast-spiking neurones (which fire at a rate of over 100 Hz), are of different cellular morphologies (small round and bitufted) and are found in most cortical layers. The final class of cell are irregular-spiking neurones, which have an unpredictable inter-spike interval, again have a varying morphology and are found in all layers except layer II/III.

The two main cell types in layer II/III (regular- and late-spiking) were shown to have different integrational properties in response to prolonged activation *via* superficial synapses (Beggs *et al.*, 2000, figure 1.5.6). Regular-spiking neurones reached firing threshold soon after the start of a 20 Hz train of stimulation, but rapidly showed accommodation. In contrast, late-spiking cells showed a delay in reaching firing threshold and then maintained firing during the remainder of the repetitive stimulation.

It is possible that such a short-term modification of the pattern of action potential generation in the perirhinal cortex may directly underlie some cognitive processes within this brain region. Indeed, computational studies based on firing patterns within a series of such neurones has shown that stimulus representations could be encoded within the perirhinal cortex in the order of seconds to tens of seconds (Tieu *et al.*, 1999; McGann & Brown, 2000). While the short-lasting changes reported here may not fully explain the functions of the perirhinal cortex, one can envisage that a complete, complex network of neurones could encode longer-term memory. Furthermore, a longer-term change in firing rate though a synaptic mechanism may indeed be the cellular mechanism for perirhinal cortex cognitive processes.

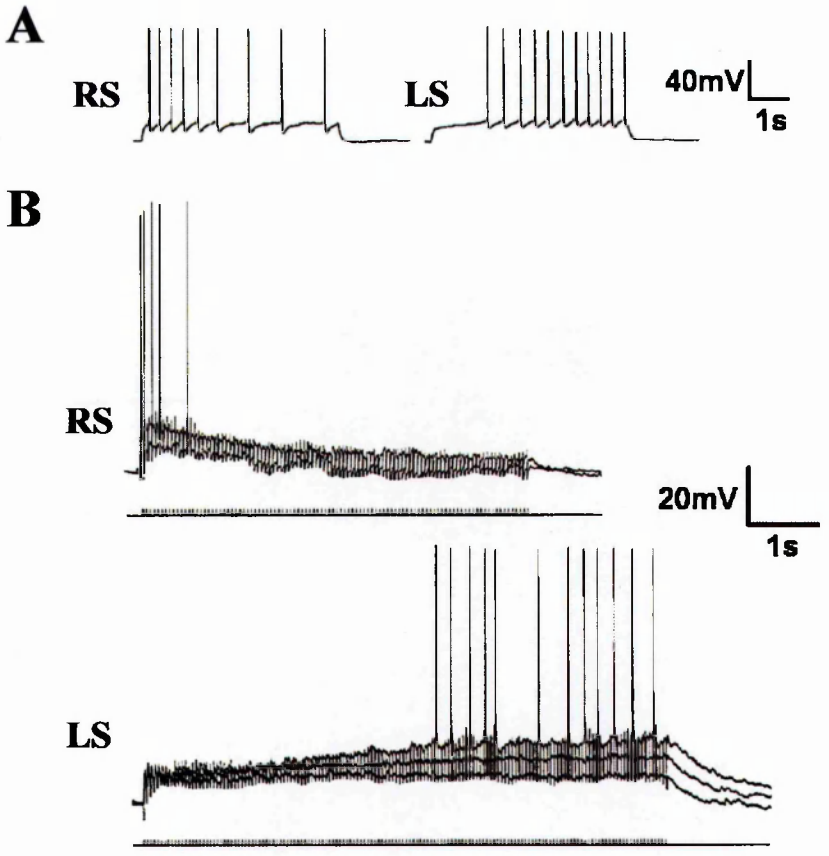


Figure 1.5.6: Regular- and late-spiking pyramidal cells of the perirhinal cortex. *A*: Regular-spiking (RS) neurones typically fire shortly after the passing of a depolarising current step and demonstrate accommodation. Late-spiking (LS) neurones fire at a longer latency and do not accommodate. *B*: RS and LS cells respond differently to trains of synaptic inputs (20 Hz). Increasing stimulation intensities are shown in both cases. RS neurones fire action potentials soon after the onset of the train of stimuli, and then accommodate. In contrast, LS neurones show a long delay before firing and then maintain action potentials for the remainder of the train. (Adapted from Beggs *et al.*, 2000)

1.6 Cortical synaptic plasticity in HD

As discussed in section 1.4, the majority of studies of synaptic plasticity within Huntington's disease have concentrated on either the striatum (being the most affected brain region in HD) or the hippocampus (being the most commonly studied region generally in electrophysiology). Therefore very little is known about the changes that occur at cortical synapses during the progress of the disease. The studies that have been carried out to date have concentrated on altered basal transmission rather than synaptic plasticity *per se* and these, along with other studies, may be used to gain insight into potential changes that may occur to cortical synaptic plasticity in HD.

1.6.1 Altered receptor expression

Neurotransmitter receptor mRNA levels, protein expression and ligand binding has been found to be reduced in both the human HD the R6/2 brain (see table 1.4.1). In particular the metabotropic receptors appear to be susceptible, with mGlu, dopamine, cannabinoid, 5-HT, histamine, and noradrenalin receptors showing a decline in the striatum, a pattern that is mirrored in the cortex for those receptors that have been assessed in this region. In particular, the R6/2 striatum shows a massive reduction in both D_1 and D_2 receptors, with as low as 33% expression compared to wild-types (Cha *et al.*, 1998; 1999). While these authors do not report expression levels in the cortex, preliminary data from the Levine lab indicates a decrease in both receptor

subtypes (Ariano *et al.*, 2001). Given that there are reductions in the receptors involved in synaptic plasticity (if only in a modulatory role), it is likely that cortical plasticity could be affected.

Protein trafficking could also affect receptor expression. As discussed in section 1.2.6, both huntingtin and huntingtin interacting proteins are involved in vesicle trafficking which has been shown to be defective in HD. HIP1 deficient mice show a dose-dependent defect in endocytosis of AMPA receptors. Given that AMPA receptor trafficking is thought to be important in synaptic plasticity, particularly expression of LTD, it is possible that plastic changes could be affected by this defect.

1.6.2 Altered NMDA receptor activity

In particular it has been demonstrated that pyramidal neurones within the frontal cortex (and striatum) of both R6/2 and knock-in mice show an enhanced sensitivity to NMDA, as demonstrated by enhanced NMDA-induced cell swelling (Levine *et al.*, 1999). Cell-swelling is thought to be an early indication of neurotoxicity and cell death (Dodt & Zieglgänsberger, 1990, 1994; Colwell *et al.*, 1996; Colwell & Levine, 1996). The enhanced cell swelling observed within the medium-sized spiny neurones within the striatum of these transgenic mice are associated with increased NMDA-mediated currents (Levine *et al.*, 1999; Laforet *et al.*, 2001) and there is evidence this could be specific to the NR2B subunit (Chen *et al.*, 1999b; Zeron *et al.*, 2002). It is possible that NMDA receptors in the cortex could demonstrate a similar enhancement, thereby altering calcium currents important in induction of synaptic plasticity.

1.6.3 Altered glutamate uptake

Alterations in glutamate uptake have been reported in R6 mouse cortex. Glutamate transporters are important for the maintenance of the low-level of glutamate in the extracellular matrix that is required for effective glutamatergic synaptic transmission and the prevention of excitatory amino acid-induced excitotoxicity (Danbolt, 1994). Glutamate transporters can be either located directly on the neurones themselves or on astroglia. Following uptake, glutamate is metabolised by glutamine synthetase into the non-toxic amino-acid glutamine. Glutamine is then returned to the neurone to be converted back to glutamate and made available for neurotransmission (Yudkoff *et al.*, 1993). Both messenger RNA and protein expression of the astrocyte glutamate transporter, GLT1, have been shown to be significantly reduced in an age dependent manner within the cortex (and striatum) of both the R6/2 and R6/1 transgenic mouse lines (Liévens *et al.*, 2001). Moreover, D-aspartate binding, which is a measure of glutamate uptake (Danbolt, 1994), is also decreased in the R6/2 cortex (and striatum) at 12 weeks, but not 4 weeks (Liévens *et al.*, 2001). Decreased glutamate uptake within the cortex could result in glutamate remaining within the synapse for a longer period and, hence, potentially increased postsynaptic receptor activation or, more drastically, glutamate-induced cell death. This effect could be compounded further by the increased activity of the NMDA receptor discussed above.

1.7 Summary

Huntington's disease is an incurable neurodegenerative disease caused by an expanded CAG repeat in the gene encoding the protein huntingtin. Classically characterised by loss of motor control and dementia, it is now appreciated that a subtle cognitive decline is a common feature of the early stages of the disease. At this stage patients are otherwise asymptomatic, including no overt neurological problems or dementia, or indeed cell death. In particular recognition memory is affected early in the progress of HD. Recognition memory is thought to be encoded within the perirhinal cortex by a mechanism involving the phenomenon of long-term depression. Synaptic plasticity is known to be perturbed in the striatum and hippocampus of different mouse models of HD. It is not, however, known what affect expression of the mutant gene has on cortical plasticity. This issue will be addressed within this thesis by employing the R6/1 mouse model to assess synaptic plasticity within the perirhinal cortex.

1.8 Aims

There has been no investigation to date of the effects that the gene responsible for HD has on cortical synaptic plasticity. This is an important issue as emerging evidence suggests that the earliest symptoms of HD may be attributable to alterations in cortical function. The aim of the work presented here is to address this question, using the R6/1 mouse model of HD.

Defects in recognition memory are apparent in HD gene carriers several years, sometimes decades, prior to the onset of the classical symptoms. One of the key cortical regions involved in recognition memory is the perirhinal cortex and it is this region which was investigated in this study. Perirhinal cortical synaptic plasticity has been well characterised in the rat, there have been no electrophysiological studies of the mouse perirhinal cortex. Therefore, in order to assess changes in the R6/1 mouse model of HD, the first part of this thesis characterises the electrophysiological properties of perirhinal cortical neurones and synapses in the normal mouse. The second part is a linear age study of the electrophysiological properties of the perirhinal cortex in R6/1 transgenic mice and age-matched littermates. Finally, immunohistochemical techniques are used to identify some of the cellular pathological changes that may underlie the changes observed in the electrophysiological study.

2. Materials and Methods

The majority of my research is based within the field of *in vitro* electrophysiology, using a mixture of both field and sharp electrode techniques. The results obtained from these experiments have been complement with histological applications to assess neuropathological changes within the cerebral cortex of the R6/1 mouse model of Huntington's disease.

2.1 Electrophysiology

2.1.1 Coronal slice preparation

Perirhinal slices were prepared from CBA × C57BL/6 mice or R6/1 mice and their wild type littermates. Despite obvious weight differences between male and female R6/1 mice (see figure 3.3.1), animals of both sexes were used throughout this study. Animals of the appropriate age were killed by cervical dislocation and decapitation in accordance with U.K. legislation (Animal (Scientific Procedures) Act 1986). The skull was exposed by cutting the scalp along the midline of the head with a scalpel. The skull was then cut along the midline using a pair of fine surgical scissors and removed with a pair of curved forceps. The meninges were carefully removed without damaging the underlying brain. A micro-spatula was then used to scoop the brain from the remaining skull and was then chilled in ice-cool (1-4°C) dissection artificial cerebrospinal fluid (ACSF; comprising, in mM: 124 NaCl, 3 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 10 D-glucose, 10 $MgSO_4$ and zero calcium)

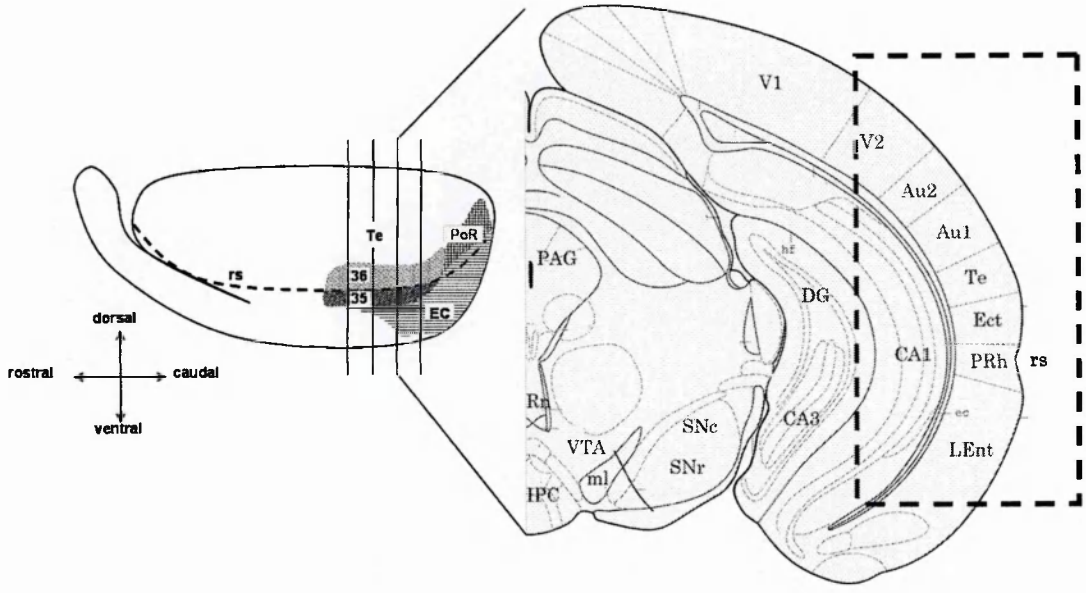


Figure 2.1.1: Coronal brain slice. *Left,* Schematic representation of the lateral view of the mouse brain. 400 μm coronal slices were cut from the area of cortex marked by the horizontal lines. *Right*, Hemispheres of the brain were trimmed to include the area marked by the dotted box. Abbreviations: Au1/2, primary/secondary auditory cortex; CA1/3, fields of cornu ammonis; DG; dentate gyrus; ec, external capsule (corpus callosum); EC, entorhinal cortex; Ect, ectorhinal cortex; hf, hippocampal fissure; IPC, interpeduncle nucleus; LEnt, lateral entorhinal cortex; ml, medial lemniscus; Rn; Raphe nucleus; rs, rhinal sulcus; PAG, periaquiductal grey; SNc substantia nigra pars compacta; SNr substantia nigra pars reticula; V1/2, primary/secondary visual cortex; VTA; ventral tegmental area. (Right-hand image adapted from Paxinos & Franklin, 2001.)

bubbled with 95% O_2 and 5% CO_2 (Carbogen; British Oxygen Company). The cerebellum, hindbrain, a section of the forebrain and a section of the ventral portion of the brain were then removed using single razor blade cuts. Excess ACSF was removed from the rostral end of the brain with a filter paper (Whatman hardened circles) and the brain fixed to a silicone stage with cyanoacrylate glue (Radio Spares, Corby, United Kingdom). The silicone stage was then placed in a cutting carriage and submerged in ice-cool dissection ACSF and continuously bubbled with Carbogen. Coronal sections (400 μm in thickness), containing the perirhinal cortex, were cut with a manual vibrotome (Campden Instruments Ltd., Sileby, UK). The rhinal sulcus and the shape of the underlying hippocampus were used as anatomical markers for the perirhinal cortex. Slices were then bisected into separate hemispheres and each trimmed to leave the perirhinal and adjacent temporal (dorsal side) and entorhinal/ectorhinal (ventral side) cortices as shown in figure 2.1.1.

2.1.2 Oblique slice preparation

Previous studies carried out in the rat perirhinal cortex utilised slices cut at a 45° oblique angle to the dorsoventral axis. Therefore this preparation was also investigated for mouse perirhinal cortical slices. The brain was removed as described above and 45° oblique cuts were made, as shown in figure 2.1.2 and the brain glued to the silicone stage. 400 μm slices were cut and trimmed to leave the perirhinal and surrounding cortices.

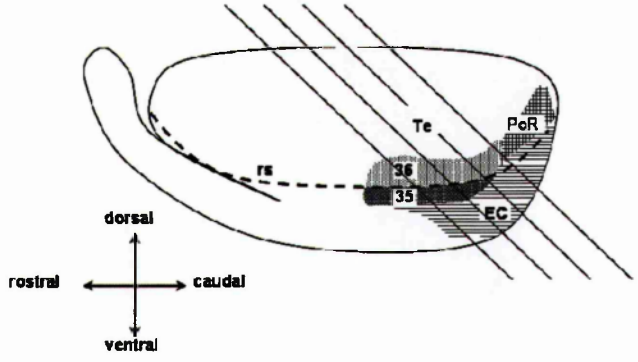
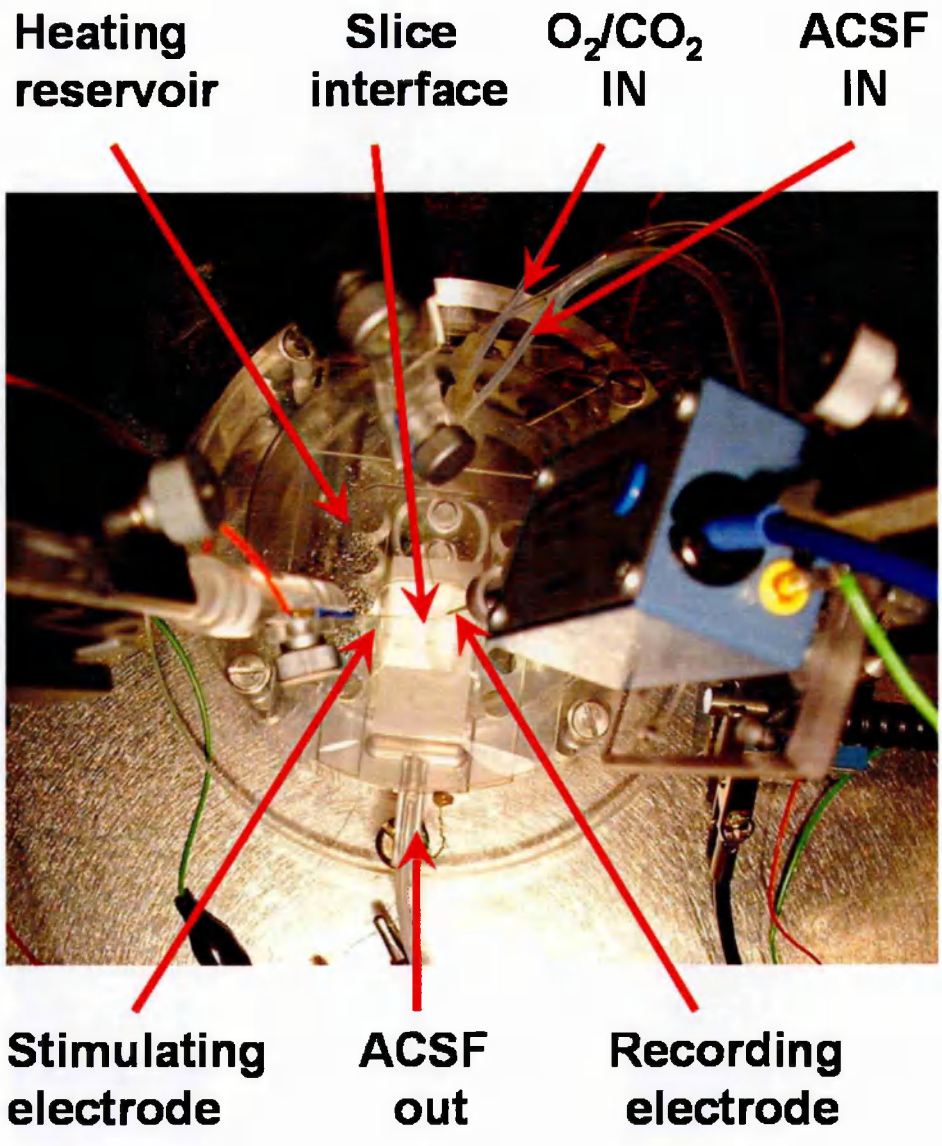


Figure 2.1.2: Oblique slices. 400 μm 45° oblique slices were cut from the region of cortex marked by the diagonal lines. (See figure 2.1.1 for abbreviations)



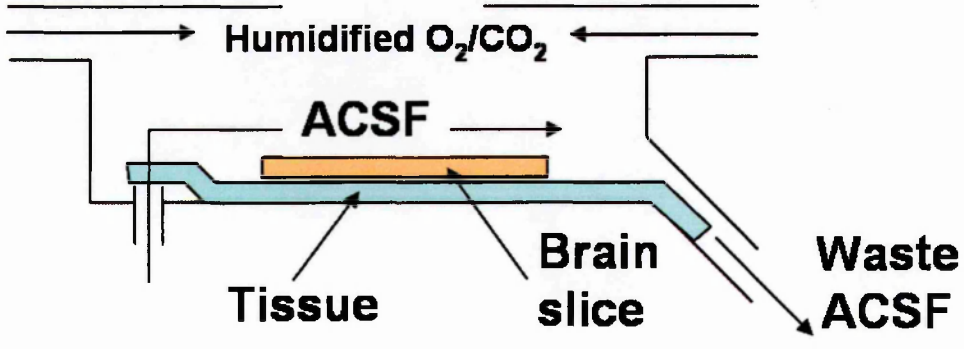


Figure 2.1.3: The interface chamber. *A*, Photograph of the interface recording chamber and electrodes positioned in the slice. *B*, Schematic of the interface chamber. Oxygenated ACSF was pumped into the chamber at a flow rate of ~125 μl/min and heated to 28°C. A thin layer of ACSF formed over the slice and was then removed to waste. The region immediately above the slice was supplied with a constant flow of humidified Carbogen (95% O_2/5% CO_2).

2.1.3 Interface recording chamber

Following preparation, slices were transferred to an interface recording chamber (Scientific Systems Design Inc., Mercerville, NJ, U.S.A.; figure 2.3), lined with a double layer of lens cleaning tissue (Whatman plc. Brentford, U.K.), using a sable bristle watercolour paint brush (size 4; H. G. Rant, High Wycombe, U.K.). Standard ACSF (as dissection ACSF with 2 mM $CaCl_2$ and 1 mM $MgS0_4$), bubbled with Carbogen, was continuously pumped into the chamber at a flow rate of approximately 125 $\mu l.min^{-1}$ (Watson-Marlow Ltd., U.K.) and heated (Proportional Temperature Controller; Scientific Systems Design Inc., Mercerville, NJ, U.S.A.) to 27°C in a water jacket before reaching the slice. A layer of ACSF formed over the slice before being pumped away to a waste reservoir. The space above the slices was supplied with humidified Carbogen. The chamber was illuminated using a cold-light source (KL1500LCD; Schott UK Ltd. Stafford, U.K.). Slices were allowed to recover for one and a half hours prior to experimentation.

2.1.4 Extracellular recording

Extracellular recording electrodes were pulled from borosilicate glass capillaries (Harvard Apparatus Limited, Edenbridge, U.K.) using a horizontal micropipette puller (Flamming/Brown Micropipette Puller P87, Sutter Instruments Corp., Novato, CA, U.S.A.) and filled with 1 M sodium chloride and 2% pontamine sky-blue 5BX (electrode impedance 4-8 MΩ). Recording electrodes were connected to an

Axoclamp 2B DC-preamp amplifier (Axon Instruments Inc., Foster City, CA, U.S.A.), *via* an Axoclamp head-stage (gain: ×0.1), by sliver/silver chloride wire. By using a coarse control micro-manipulator, the recording electrodes were positioned in layer II/III of the perirhinal cortex directly below the rhinal sulcus (see figure 2.1.4). The circuitry for electrophysiological recording is shown in figure 2.1.5.

2.1.5 Intracellular recording

Intracellular recording electrodes were pulled from borosilicate glass capillaries (Harvard Apparatus Limited, Edenbridge, U.K.) on a horizontal micropipette puller (Flamming/Brown Micropipette Puller P87, Sutter Instrument Corp., Novato, CA. U.S.A.) and filled with 3 M potassium acetate (electrode impedance 60-140 MΩ). Electrodes were connected to an Axoclamp 2B head-stage (gain: ×0.1) by a silver/silver chloride wire. The head-stage was attached to a micro-manipulator and in addition attached to a fine control micro-driver (Newport Components Ltd., C&D Technologies Inc. U.S.A.), allowing stepped advancements of 2 μm through the slice. Electrodes were positioned at layer II/III of the perirhinal cortex, directly below the rhinal sulcus (figure 2.1.4) and brought into contact with the slice as indicted by the formation of a closed circuit. The head-stage was connected to an Axoclamp 2B DC-preamp amplifier (Axon Instruments Inc., Foster City, CA, U.S.A.) operated in bridge mode (current clamp). See figure 2.1.5.

2.1.6 Stimulating electrodes

Stainless steel, monopolar stimulating electrodes (A-M Systems, Carlborg, WA, U.S.A.) were positioned approximately 150-350 μm from the recording electrode in either layer I or layer III to study the superficial and intermediate pathways respectively (figure 2.1.4). Responses were evoked by constant current stimulation (1-600 μA, 40 μs). In some experiments two electrodes were placed either side of the recording electrode. This setup allowed the stimulation of two independent pathways, thus enabling the homosynaptic nature of long-term synaptic plasticity to be assessed within the perirhinal cortex. Stimulating electrodes were connected, *via* Neurolog isolated stimulators (NL800, Digitimer Ltd., U.K.), to an IT-16 computer interface digital-analogue converter (Instrutech Corp., NY, U.S.A.). Coarse control micro-manipulators were used to position the stimulating electrodes at the appropriate locations.

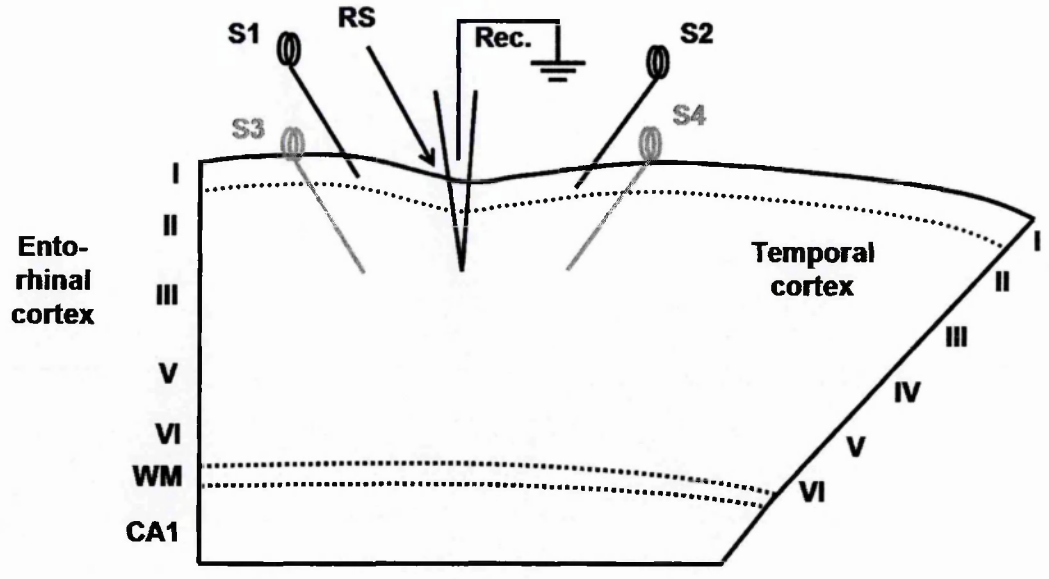


Figure 2.1.4: Positioning of stimulating and recording electrodes within a perirhinal slice. Cortical layers are indicated (note the absence of layer IV on the entorhinal side of the perirhinal cortex). A recording electrode was positioned in layer II/III directly below the rhinal sulcus. Stimulating electrodes were positioned either side of the sulcus either in layer I (superficial pathways; S1 & S2) or within layer III (intermediate pathway; S3 & S4).

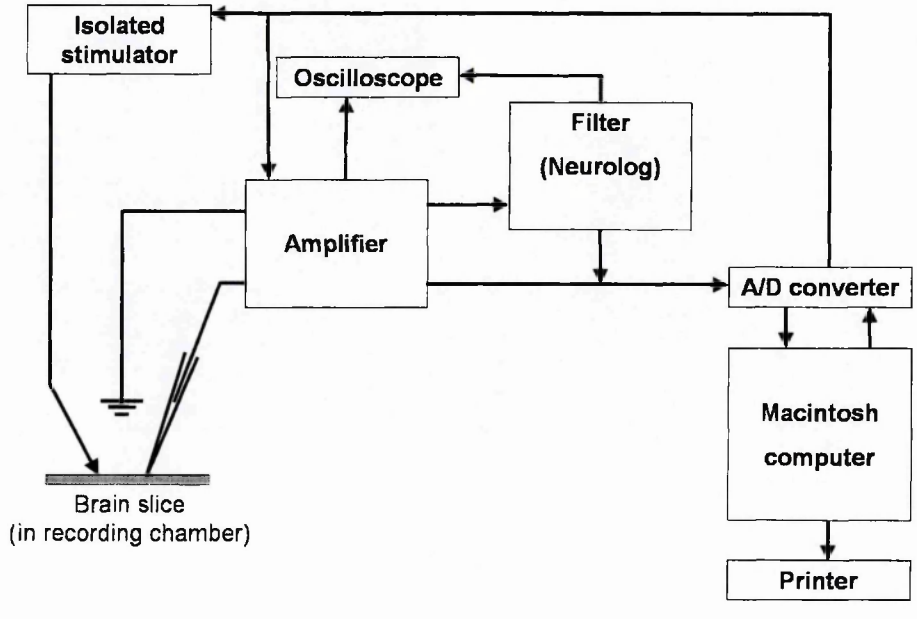


Figure 2.1.5: Basic electrophysiological recording set up. Recording electrodes were positioned in the perirhinal slice and connected to an Axoclamp 2B DC-preamp amplifier or a Neurolog AC-preamp amplifier. The electrical signal was then passed simultaneously to an oscilloscope and, *via* an A/D converter, to a Macintosh computer. Electrical stimuli were controlled by the computer and applied to the slice though an isolated stimulator box.

2.1.7 Prevention of mechanical and electrical interference.

To reduce mechanical disturbance, the recording chamber and micro-manipulators were placed on an anti-vibration air-table (Technical Manufacturing Corp., Peabody, MA, U.S.A.). All electrical apparatus was earthed to one central point. In addition, the room was electrically shielded to prevent electrical noise from external sources.

2.1.8 Stimulation paradigms

2.1.8.1 Extracellular recordings

Synaptic responses

Constant current stimulation was applied though monopolar stimulating electrodes to layer I or III of the cortex. The duration of the stimulus was set to 40 μs.

Input/output relationship

To ascertain the maximum amplitude of the synaptic response test shocks were applied at increasing stimulus intensities between 10 and 600 μA.

Paired-pulse stimulation

Stimulus intensity was set to evoke responses at 30, 50 and 80% of the maximal amplitude. Two identical test shocks were applied at intervals between 20 and 2000 ms and the ratio of these responses were calculated as a percent change:

$$\left(\frac{P1}{P2} \times 100\right) - 100$$

Pathway independence

In experiments where two stimulating electrodes were placed, pathway independence was determined by ensuring no heterosynaptic depression occurred. A series of stimulation paradigms were used to assess this. Firstly, homosynaptic paired-pulse depression was confirmed at an interval of 100 ms in each separate pathway. Secondly, the absence of heterosynaptic depression was confirmed by applying a test shock to the second pathway at a delay of 100 ms after the first. Finally, test shocks were applied to both pathways simultaneously to ensure summation occurred, further demonstrating the independent nature of the pathways. If pathway independence was not evident then the electrodes were re-positioned and independence confirmed.

Long-term potentiation

Stimulus intensity was set to evoke responses at 30% of the maximal amplitude. Synaptic potentials were evoked at 0.033 Hz and collected for 15-20 minutes. Following a stable baseline, theta-burst stimulation was applied. TBS comprised of four trains applied every 15 seconds. Each train consisted of 10 bursts of 10 pulses

at 100 Hz, (inter-burst interval 200 ms) applied at test shock intensity. Following TBS, responses were recorded for 1 hour at a frequency of 0.033 Hz.

Long-term depression

Stimulus intensity was set to evoke responses at 80% of the maximum amplitude. Test shocks were applied every 30 seconds (0.033 Hz) and synaptic potentials were recorded for 15-20 minutes. Low-frequency stimulation (LFS) was then applied to induce LTD. LFS consisted of 900 pulses at 1 Hz applied at test shock intensity. Following LFS, responses were monitored every 30 seconds for at least 1 hour.

2.1.8.2 Intracellular recordings

Neuronal impalement

The micro-driver was used to slowly step the intracellular recording electrode through the slice. Neuronal impalement was achieved by a brief pulse of positive current (40-60 nA, <2 ms) or a brief period of over-compensated capacitance. Spontaneous action potentials were often observed upon initial impalement which ceased upon an immediate ramped negative current (typically approximately –1 nA) applied to the cell. As the neurone stabilised, the negative current was slowly removed. Once fully stabilised, healthy neurones were selected on the basis of a stable membrane potential and an action potential with an overshoot greater than 60 mV above firing threshold. Neurones failing to meet these criteria were discarded.

Passive membrane properties

Once the cells were stabilised passive membrane properties were measured. Current-voltage (I/V) relationships were measured by intracellular injection of hyperpolarising and depolarising constant current pulses (–0.8 nA to +0.8 nA, 150 ms). Action potentials were evoked upon the application of depolarising current. Membrane properties of the neuronal cells were determined, including threshold potential, action potential amplitude and width at half way between threshold and maximum potential. The gradient of the I/V plot in response to hyperpolarising current was used to estimate the input resistance (R_{in}) of each neurone. Membrane capacitance (C_m) was calculated from the membrane input resistance and the time constant (τ_m) using the following relationship: $C_m = \tau_m / R_{in}$. Values of τ_m were obtained using the protocol in Bindman *et al.* (1988) as follows: Semi-logarithmic plots were made of the voltage response against time during the charging of the cell by hyperpolarising current pulses. The charging response was always well fitted by a single exponential line (R^2 value greater than 0.99) and the time constant calculated as the negative reciprocal of the best-fit line at $(1-1/e \times 100)\%$ of the maximum deflection.

Synaptic responses

Intracellular EPSPs were evoked by constant current stimulation (1-200 μA, 40μs) *via* monopolar stimulation electrodes positioned in layer I of the cortex, either ventral or dorsal to the recording electrode. To ensure that a pure EPSP was recorded a

subthreshold EPSP was evoked during the steady-state period of both a hyperpolarising and depolarising current step. Early experiments showed that there was indeed an inhibitory component to the recorded response. It was found that a low concentration (2.5 μM) of $GABA_A$ antagonist picrotoxin (PTX, Curtis *et al.*, 1970), added to the ACSF, was required to remove this component. Therefore in all intracellular experiments 2.5 μM PTX was present.

Long-term depression

Induction of long-term depression of the intracellularly recorded response was attempted by the application of various protocols including 900 pulses applied at 1 Hz, 300 pulses applied at 1 Hz and also pairing synaptic activity with a depolarising current to the cell.

2.1.9 Pharmacology

Drugs were dissolved in standard ACSF at the appropriate concentration and applied by perfusion into the chamber in place of the standard ACSF. Drugs reached the chamber within 5-10 minutes and were then allowed a minimum of 30 minutes to equilibrate within the chamber before electrophysiological manipulations were carried out. If available, water soluble drugs were always obtained. Where suitable water soluble drugs were not available, the effect of the appropriate vehicle (e.g. ethanol, dimethylsulphoxide (DMSO), sodium hydroxide (NaOH)) was assessed on basal transmission, paired-pulse and long-term depression prior to experimentation.

2.1.9.1 List of drugs used

Drugs supplied by Tocris-Cookson Ltd. (Avonmouth, U.K.):

UPF 523/(RS)-1-aminoindan-1,5-dicarboxylic acid (AIDA; group I mGlu receptor antagonist); 6-cyano-7-nitroquinoxaline-2, 3-dione disodium (CNQX; AMPA/kainate receptor antagonist); (RS)-α-cyclopropyl-4-phosphonophenylglycine (CPPG; group III mGlu receptor antagonist); D-(–)-2-amino-5-phosphonopentanoic acid (D-AP5; NMDA receptor antagonist; also supplied by Sigma-Aldrich, see below); (2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495; group II mGlu receptor antagonist); 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059; MAP kinase kinase inhibitor); Quinpirole hydrochloride (D_2 dopamine receptor agonist); (R)-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride (SCH23390; D_1 dopamine receptor antagonist); (2S)-(+)-5,5-dimethyl-2-morpholineacetic acid (SCH50911; $GABA_B$ receptor antagonist); (±)-1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol hydrobromide (SKF38393; D_1 dopamine receptor agonist); Remoxipride hydrochloride (D_2 dopamine receptor antagonist).

Drugs supplied by Sigma-Aldrich Company Ltd. (Poole, U.K.):

(–)-bicuculline methiodide (BMI; $GABA_A$ receptor antagonist); D-(–)-2-amino-5-phosphonopentanoic acid (D-AP5; NMDA receptor antagonist; also supplied by Tocris, see above); dimethylsulphoxide (DMSO; solvent); picrotoxin (PTX; $GABA_A$

receptor antagonist); Pontamine sky-blue 5-BX (dye); Sodium hydroxide (NaOH; solubility aid); (–)-sulpiride (D_2 dopamine receptor antagonist).

2.1.10 Analysis

Responses were visualised on a digital oscilloscope (Tektronix Inc., Beaverton, OR, U.S.A.), collected on an Apple Macintosh computer and analysed using A/DVANCE 3.6 software (Robert McKellar Douglas, Vancouver, Canada). The amplitude of the synaptic component of the recorded potentials was measured as the difference in mV between a baseline value and the peak amplitude. Maximum slope was calculated in $V.s^{-1}$ from the initial deflection of the synaptic component of the recorded potential. The area of the synaptic response was measured in picovolt seconds (pVs) from the initial synaptic component to the end of the recording period. All measurements were made by the manual placement of cursors. Data were transferred to Microsoft Excel 1998 or 2002 for further analysis.

In experiments looking at the outcome of paired-pulse stimulation, the second response was expressed as a percentage change from the first (P2/P1×100)–100. For summary figures a "composite paired-pulse ratio" was produced by summing the individual ratios obtained at all paired-pulse intervals. Changes in the composite paired-pulse ratio represent differences in the overall profile, but do not identify specific changes at a given interval. Therefore, for statistical analysis two-way ANOVA were carried out to verify effects of both condition (e.g. drug or phenotype) and interval.

In experiments designed to investigate long-term changes in synaptic efficacy, responses were normalised to the last 10 minutes of baseline prior to conditioning and expressed as a percentage change from baseline. LTP was defined as a stable increase of greater than 10% and LTD as a stable decrease of greater than 10% in the synaptic potential, 50-60 minutes after the end of the conditioning period. Data for each condition were pooled together, irrespective of experimental outcome and are expressed as mean ± standard error of the mean (SEM):

$$\frac{\text{standard deviation}}{\sqrt{(n-1)}}$$

Graphical representations were produced in Microsoft Excel 2002 and presented in Microsoft PowerPoint 2002 or Adobe Photoshop 7.0 (2002). Student t-tests, Welch corrected t-tests and one-way ANOVA were carried out using InStat 3.05 (GraphPad Software Inc., 2000). Two-way ANOVA were carried out using Statistica 6.0 or 6.1 (StatSoft Inc., 2002/2003).

Both slice and animal n values are reported in the following format: n= number of slices (number of animals). Statistical significance is indicated in summary charts as follows: * = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$.

2.2 R6/1 transgenic mice

2.2.1 Breeding

Founder mice for the Open University R6/1 transgenic mouse colony were obtained from the University Laboratory of Physiology, Oxford University, U.K., courtesy of Drs. Anthony Hannan and Anton van Dellen. Mice transgenic for exon 1 of the mutated human gene responsible for Huntington's disease were then bred in-house by backcrossing transgenic males to CBA × C57BL/6 females (Open University colony). Maintenance and care of the mouse line was carried out by Dawn Saddler.

2.2.2 Genotyping

Genotyping was carried out by Ph.D. students Austen J. Milner and Sarat C. Vatsavayai in our lab and in strict accordance with the procedure described in the Dr. Murphy HD Project Licence. A brief overview of the method is provided below.

2.2.2.1 Tissue retrieval

For identification purposes, mice were ear-marked upon weaning using a small punch and then tail tipped to provide tissue for genotyping. Tail tipping was carried out following the application of spray local anaesthetic (ethyl chloride; Roche Products Ltd.) to the tail. Approximately 5 mm was then severed using scissors and

forceps (cleaned in 99.9% alcohol between each animal). The tip was transferred to a 500 μl Eppendorf® vial and stored at + 4°C until DNA extraction was carried out.

2.2.2.2 DNA extraction

250 μl of lysis buffer was added to each tissue sample and incubated overnight at <50°C (proteinase K rapidly denatures at temperatures exceeding 60°C). 125 μl saturated sodium chloride solution was then added and agitated vigorously by hand for 5 minutes and then microcentrofuged at 1300 RPM for 30 minutes. 325 μl of 100% ethanol was added to empty sterile Eppendorf tubes and the supernatant from the original samples were then added to the new vials. A DNA flocculent formed, which was then pelleted by centrifugation at 1300 RPM for 10 minutes. The supernatant was removed and 300 μl of 70% ethanol added and re-centrifuged for one minute and the ethanol removed and allowed to air dry for at least 15 minutes. The DNA was then resuspended in 30 μl TE overnight at room temperature.

2.2.2.3. Polymerase chain reaction

A master mix was prepared in a sterile Eppendorf® tube and mixed thoroughly. 1 μl of sample DNA was added to a tube and as controls R6/1 DNA and PCR H_2O was added to two other tubes. 9 μl of the master mix was then added to each tube, which were then loaded into a Hybaid Omni PCR machine. The following PCR cycle was then used:

1) 94°C for 2 minutes

2) 94°C for 30 seconds

3) 65°C for 30 seconds

4) 72°C for 45 seconds

5) Steps 2-4 repeated for 36 cycles

6) 72°C for 10 minutes

2.2.2.4. DNA detection

The transgene was identified following gel electrophoresis in ethidium bromide-agarose. 2.5 μl loading buffer was added to the PCR products and then 6 μl of each sample was loaded onto a 1.5% agarose gel submerged in 1xTBE. At either end of the gel a 1 kb DNA ladder was also loaded. The gel was run at 100 Volts for 30-45 minutes and the bands were visualised under a fluorescent gel reader. The presence of the transgene was indicated by a band at 272 bp.

2.2.2.5. Solutions for genotyping

Saturated sodium chloride solution

Approximately 5 M solution was made up in deionised water.

Lysis buffer

Lysis buffer consisted of 50 mM Tris-HCl at pH 8.0, 100 mM EDTA and 0.5 % SDS made up in deionised water.

Proteinase K

To purify the target material from contaminating proteins proteinase K was used. This is a non-specific endolytic protease that cleaves peptide bonds at carboxylic sides of aliphatic, aromatic or hydrophobic amino acids. Proteinase K was obtained from either Fisher Scientific U.K. (Loughborough, U.K.) or Sigma-Aldrich Company Ltd. (Poole, U.K.).

TE

A Tris-EDTA buffer at pH 7.0 – 8.0 was used to reduce hydrolysis of bases by chelating divalent cations.

Master mix

Each 10 μl reaction consisted of 3.35 μl PCR H2O, 1 μl 2 mM dNTPs, 1 μl forward primer, 1 μl reverse primer, 1.5 μl Expand^{TM} 10× Buffer (with Mg^{2+}), 1 μl DMSO and 0.15 μl Expand^{TM} polymerase.

Forward primer

5'-3': CGC AGG CTA GGG CTG TCA ATC ATG CT (26 bases)

Reverse primer

5'-3': TCA TCA GCT TTT CCA GGG TCG CCA T (25 bases)

2.3 Behaviour

Limb-clasping

The limb-clasping aspect of the R6/1 phenotype was assessed by tail suspension. Mice were considered as positive for clasping if they demonstrated this trait in three or more out of ten trials. *Percentage* of mice displaying limb-clasping was then plotted against age.

2.4 Histochemistry

Histochemical techniques were used to visualise pathological changes within the R6/1 mouse brain. A haematoxylin and eosin (H & E) stain was used to highlight the cellular architecture of the perirhinal slice. Immunohistochemistry, using a primary antibody targeted to N-terminal mutant huntingtin, was used to investigate aggregate development within the R6/1 brain. Thirdly, fluorescent labelling of antibodies raised to D_1 and D_2 dopamine receptors was used to quantify progressive changes in the expression of these receptors.

2.4.1 Section preparation

In order to visualise sections of perirhinal cortex a variety of procedures were employed to provide tissue. Initially mice were fixed by intracardial perfusion of 4% paraformaldehyde (PFA). Perfusions were performed by either Mark Eyre or Christopher Peddie following anaesthesia with Sagatal.

It was found that submerged-fixed brains do not show overt differences from perfusion-fixed brains when visualised at the light-level. Following extraction of the brain from the skull (see dissection for electrophysiology) the whole brain was rinsed in ice-cold ACSF and then immediately submerged in 4% PFA for 48 hours followed by 2% PFA overnight and finally transferred to PBS.

Various techniques were attempted in the harvesting of slices from the chamber following electrophysiological analysis. Retrieval into ice-cold ACSF

followed by immersion fixation in 4% PFA for 48 hours was found to yield results similar to those obtained from perfused animals. Slices harvested from the chamber following electrophysiological analysis (6-10 hours) were found to yield poorer quality images for immunohistochemistry than those fixed directly following dissection. Therefore, to enable a direct comparison between electrophysiological and histological results, a slice that was prepared for electrophysiology was instead fixed immediately after dissection in 4% PFA for 48 hours and then transferred to PBS. Most sections in this study were prepared in this manner.

Following fixation, slices were resectioned at 50 μm. Cryostat (Leica CM1900; Leica Instruments, Germany) sections were found to be unsuitable, as the surface layers of the cortex, particularly the perirhinal region, were prone to detachment. Even cryoprotection and embedding in Tissuetech (Histological Equipment Ltd., Nottingham, U.K.) failed to provide suitable sections. Resectioning using a vibrotome (Leica VT1000s, Leica Instruments, Germany) was far superior. Sections were embedded in a high clarity agarose gel and resectioned at 50 μm on the vibrotome and collected in PBS.

2.4.2 Toluidine blue stain

In an attempt to highlight the cell bodies, several different staining techniques were investigated. The fluorescent acridine orange produced a far too intense stain that faded almost immediately. Cresyl violet revealed the cell bodies, however also faded relatively quickly. It was found that staining the neuronal cell bodies with toluidine

blue and counterstaining the cytoplasm with eosin pink was the most suitable stain and revealed the cortical layers.

The protocol used was adapted from a standard toluidine blue stain (Bodian and Lake, 1963, In Bancroft & Stevens, 1990). Sections were immersed over night in a full strength xylene solution to permeabilise the cells. Following the clearing solution, the sections were sequentially immersed in 90% and 70% propan-2-ol (2 × 5 minute washes). Cell nuclei were then stained using 0.01% (w/v) toluidine blue in phosphate-citrate buffer, applied for approximately 10 seconds followed by two washes with 90% propan-2-ol. The cytoplasm was then counter stained using 1% eosin pink (aqueous, pH 7.4) for approximately 5 seconds. Sections were then reimmersed in 90% and 100% propan-2-ol and rinsed in xylene (2 × 5 minute washes) and finally mounted onto glass slides and cover slipped using DPX mountant.

2.4.3 Inclusion mapping

To visualise the formation of inclusions within the R6/1 mouse brain a primary antibody, raised against exon 1 of the huntingtin protein and carrying 53 glutamine residues (S830 sheep polyclonal; gift from Prof. Gillian Bates, Division of Medical and Molecular Genetics, Guy's Hospital, London, U.K.), was used. 50 μm free floating sections were first rinsed in PBS before unspecific binding was blocked using 2% bovine serum albumin in TBS for 15 minutes. Endogenous peroxidase activity was then blocked using 3% hydrogen peroxide. The primary antibody (S830) was added at a 1:2000 dilution and left overnight at +4°C. Biotinylated rabbit

anti sheep IgG (H+L) (Vector Laboratories, Ltd., Peterborough, U.K.) secondary antibody was applied for one hour at room temperature. ABC elite (avidin-biotin) (Vector Laboratories, Ltd.) was prepared and added to each set of sections for 45 minutes. Sections were submerged in 0.05% diaminobenzidine for 6 minutes and then washed in distilled water. 3 × 5 minute washes in TBS were carried out between each step.

Following the DAB reaction slices were counter stained with either methyl green or aqueous eosin and then dehydrated in 70, 90 and 100% ethanol, cleared in xylene and then mounted and cover slipped using DPX mountant.

2.4.4 Dopamine receptor expression

To visualise the distribution of dopamine receptors, antibodies raised to specific subclasses were used. D_1 primary antibodies were Rabbit anti-dopamine D1A affinity purified polyclonal antibodies (Chemicon International, Temecula, CA, U.S.A.). D_2 primary antibodies were Rabbit anti-human dopamine D_2 (long and short isoforms) affinity purified polyclonal primary antibodies (Chemicon International). To determine D_2 antibody specificity the brains of D_2 deficient mice (gift from Professor M. Levine, Mental Retardation Research Center, University of California, Los Angeles, U.S.A. Kelly *et al.*, 1997; 1998) were used as a control. To highlight D_1 and D_2 dopamine receptors a fluorescently tagged antibody was used. The signal was amplified using Tyramide Signal Amplification (TSA^{TM}) kit #14 supplied by Molecular Probes. TSA provides a system whereby low intensity signals are amplified, allowing enhanced visualisation. This is achieved by conjugating the

secondary antibody to horseradish peroxidase (HRP). Tyramide residues on the fluorescent dye are then activated by the HRP activity forming a highly reactive and short-lived species. This activated dye forms a covalent bond with neucleophilic residues in the close proximity of the primary antibody/antigen site (Molecular Probes, 2003, see figure 2.4.1). The protocol underwent several adaptations from that supplied by Molecular Probes. The final protocol is as follows:

50 μm resectioned slices were washed in PBS (3 × 10 minutes) and then cells were permeabilised and unspecific binding blocked using a "blocking reagent" for two hours. Endogenous peroxidase activity was quenched using a 3% peroxidase quenching buffer for 60 minutes. Primary antibodies were then applied at the optimum concentration of 2.5 μl/ml (previously titres of 20, 10, 5, 2.5 and 1.25 μl/ml were tested), dissolved in the blocking reagent, for 3 days. A horseradish peroxidase conjugate of a goat anti-rabbit IgG secondary antibody (1:100 of the blocking reagent) was applied and left to incubate for 24 hours. This was followed by the application of the tyramide solution diluted 1:100 in amplification buffer/0.0015 hydrogen peroxide solution (supplied in the TSA kit) for 5 hours. Finally, slices were rinsed 3 times in PBS buffer (10 minutes each wash) and then transferred to acid/alcohol-washed glass slides and allowed to dry slightly. Sections were then cover slipped using Fluoromount mountant. Unless stated otherwise, slices were washed between in each step 3 × 10 minutes in blocking reagent. All incubations were carried out at +4°C. In control experiments where slices were exposed to either primary or secondary antibodies only, slices underwent all solution changes stated above, but during exposure of experimental slices to the appropriate antibody, control slices were washed in blocking reagent.

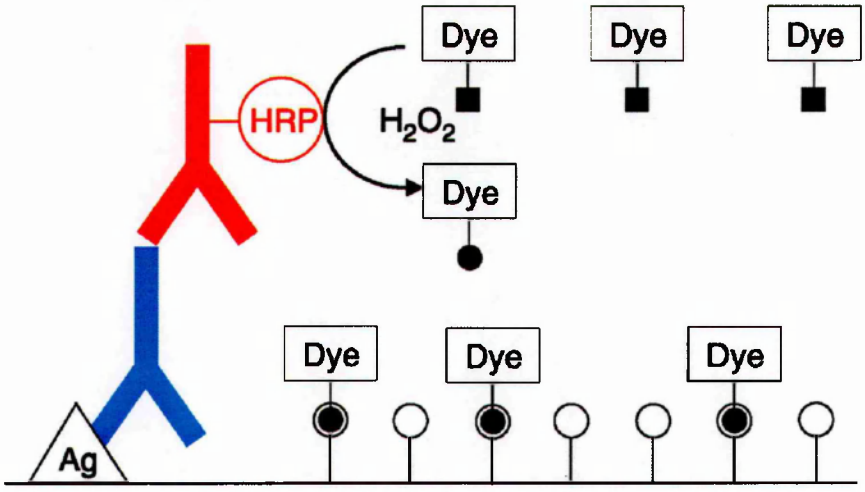


Figure 2.4.1: Tyramide Signal Amplification. Primary antibodies (Y) bind to the antigen (Ag) of interest i.e. D_1 or D_2 dopamine receptors. Horseradish peroxidase (HRP) conjugated secondary antibodies (Y) are then bound. HRP mediates the conversion of inactive (■) fluorescent dye to a short-lived active (●) form that binds to neucleophilic residues (○) in the near vicinity of the antigen. (From Molecular Probes, 2003.)

2.4.5 Histochemical solutions

Blocking Reagent (for fluorescent microscopy)

2% fish gelatine, 0.01% sodium azide, 0.1% Triton X-100 made up in 0.1 M PBS, pH 7.4 (Personal communication, Prof. Victor Popov).

Phosphate-citrate Buffer

Phosphate-citrate buffer solution was prepared according to Bancroft and Stevens (1990) and consisted of 0.2 M Na_2HPO_4 and 0.1 M citric acid at pH 4.7.

Phosphate Buffered Salt solution

0.1 M phosphate buffered saline (PBS) solution was prepared.

Tris Buffered Saline solution

500 nM Tris and ~0.09% saline solution (TBS) was prepared.

Toluidine blue

0.01% (w/v) toluidine blue was made up in a phosphate-citrate buffer, pH 4.7

2.4.5.1 Chemical sources

Chemicals supplied by Molecular Probes Europe BV, Leiden, The Netherlands:

TSA™ Kit #14 * with HRP-goat anti-rabbit IgG and Alexa Fluor ® 568 tyramide*

Chemicals supplied by Sigma-Aldrich Company Ltd., Poole, U.K.:

Acridine orange; Type VII, low gelling temperature agarose; Gelatine from cold water fish skin; 30% hydrogen peroxide solution; Paraformaldehyde; Sodium azide; Toluidine Blue, Triton-X-100.

Chemicals supplied by Prolabo, Paris, France:

Cresyl violet

Chemicals supplied by Vector Laboratories, Ltd., Peterborough, U.K.:

VECTASTAIN® Elite ABC kit; Biotinylated rabbit-anti sheep IgG (H+L);

Chemical supplied by VWR International Ltd., Lutterworth, U.K.:

DPX mountant for microscopy; Aqueous Eosin Pink; Fluoromount mountant 'Gurr'.

2.4.6 Light microscopy

Toluidine blue stained sections were visualised on a Zeiss Axiophot microscope (Carl Zeiss Ltd, U.K.) and images captured using a 35 mm colour film camera.

Sections that were prepared using S380 antibodies raised to mutant huntingtin were visualised on a Nikon Microphot-FX microscope (Nikon, Tokyo, Japan) fitted with a JVC-KY-F75V digital video camera (JVC Professional Europe Ltd, London, U.K.) and images captured onto computer using Image Pro v5.0 (Media Cybernetics, U.S.A.).

2.4.7 Confocal microscopy

Sections that were prepared by fluorescent immunolabelling were visualised using a Leica DM IBRE scanning confocal microscope (Leica Microsystems Heidelberg GmbH, Germany). A confocal microscope was used rather than a standard fluorescent microscope to reduce the level of glare from emissions in surrounding tissue, thereby increasing the definition of tissue structure. This is achieved as the confocal microscope images only a thin layer of tissue at one time; where as a standard microscope images the full thickness of the section.

Images from each experimental set of slices were taken at a standardised photomultiplier tube (PMT) intensity. The PMT threshold intensity was set to give a suitable level of fluorescence in the wild type mice, thereby maximising any decrease in intensity to be observed in the R6/1 mouse. The laser intensity was constant in all

experiments that were directly compared and to further reduce the probability of fluctuations in the laser intensity all slices were imaged on the same day.

The confocal microscope was controlled by Leica Confocal Software (Version 2.5) and images taken using the TRIT-C channel optimised for 576 nm emissions. The fluorochome (Alexa Fluor 568) was excited at 568 nm. A fixed number of images were taken in series at 0.5 μm steps and an average composite image produced. A mean fluorescence value for each cortical layer was calculated (arbitrary units) for each slice using three non-overlapping square sampling windows (mean fluorescence of a 2000 μm^2 area). Fluorescence intensity was standardised between slices by firstly imaging sections on the same day using the same lazar and PMT intensities and, secondly, by the Leica Confocal Software's internal standardisation of arbitrary units between 0 and 255. Concerns of an "edge effect" leading to artificially high fluorescent values within layer I were evaded by the positioning of sampling windows at the inner portion of layer I (see figure 2.4.2). Sampling means were then pooled to give an overall mean ± SEM for each cortical layer (maximum intensity recorded: 234.65 arbitrary units). In an attempt to remove background fluorescence, the minimum level of fluorescence was determined for the whole slice (minimum values ranged from 30 to 42 arbitrary units) and subtracted from the slice sample mean. This is an under estimation of background and, therefore, any *percent* changes highlighted in this study are, in reality, larger.

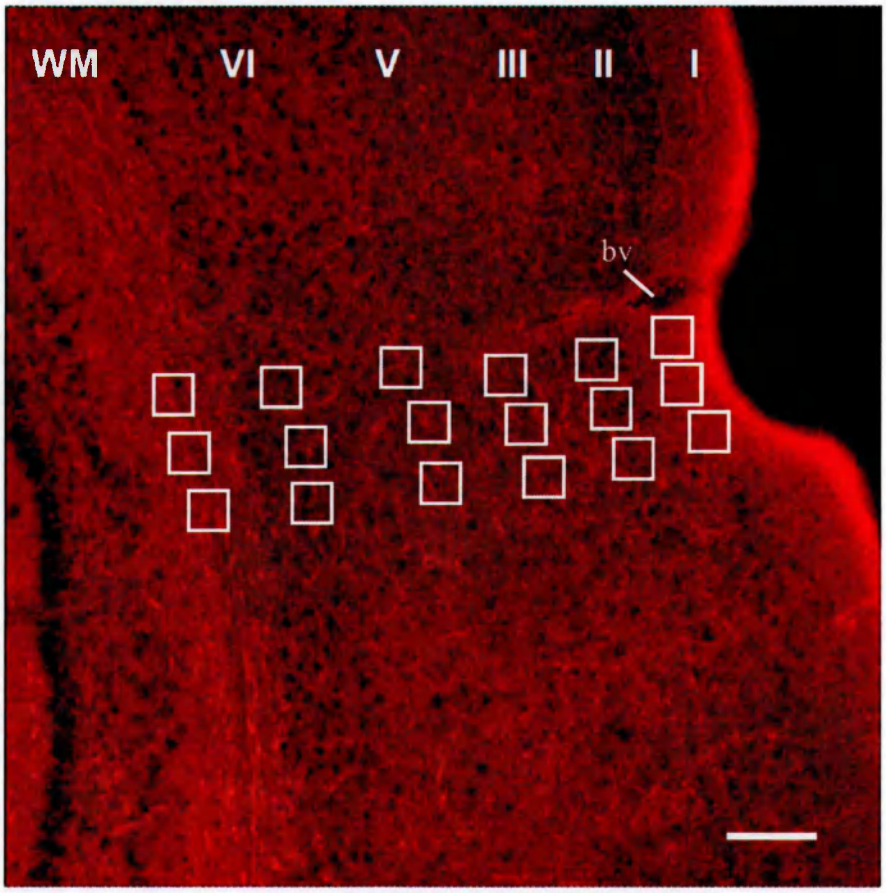


Figure 2.4.2 Example sampling windows used for fluorescence quantification.
A typical fluorescent image is shown with cortical layers indicated. The three boxes indicate typical placement of non-overlapping sampling windows within each layer. To avoid the potential "edge effect", layer I samples were taken from the border of layers I and II. Blood vessels (bv) were also avoided. Scale bar 100 μm; image obtained using a ×40 objective.

3. Results

Previous *in vitro* studies have extensively characterised long-term and short-term synaptic plasticity in the rat perirhinal cortex. There is, however, no confirmation that the mouse perirhinal cortex behaves in a similar fashion. In order to assess synaptic plasticity in the perirhinal cortex of a transgenic mouse model of HD the first part of my results will describe synaptic plasticity and transmission in the CBA × C57BL/6 mouse (the background strain of the R6/1 mice). The second part of this section uses pharmacological manipulations to investigate receptors involved in the induction of long-term depression and paired-pulse depression, again in the normal perirhinal cortex. The third part is a linear study of synaptic plasticity in the R6/1 mouse in comparison to age-matched littermates. I will then describe how pharmacological manipulation can be used to recover normal synaptic function. The final part describes the use of histological techniques to assess pathological changes in the R6/1 cortex that may underlie the differences observed in the synaptic phenotype. Throughout the results section all figures are colour coded as follows:

Control (wild-type): Blue

Drug condition (wild-type): Green

Transgenic animals: Red

Drug condition (Transgenic): Orange

3.1 Perirhinal cortical synaptic plasticity in the mouse

The aim of this section of the project was to characterise synaptic transmission and induction of synaptic plasticity in the normal mouse perirhinal cortex. Previously, LTP, LTD and short-term plasticity (paired-pulse depression) has been described extensively in the rat perirhinal cortex. It is not known, however, if the mouse perirhinal cortex behaves in a similar way. To assess this, acute perirhinal cortical slices were prepared from CBA × C57BL/6 mice and a mixture of field and sharp intracellular recordings were made.

3.1.1 Synaptic responses

Initial experiments were aimed at recreating in the mouse some of the results obtained by Bashir and colleagues in the rat, including expression of paired-pulse depression and long-term depression (Ziakopoulos *et al.*, 1999; Cho *et al.*, 2000). For these experiments mice of between 6 and 10 weeks of age were used.

3.1.1.1 Slice orientation

The rat studies were carried out using acute *in vitro* slices cut at a 45° oblique angle to the dorsoventral axis (see methods, figure 2.1.2). The same dissection applied to the mouse perirhinal cortex did not, however, yield suitable field responses. Field EPSPs were very small, with a maximum amplitude of approximately 1 mV and

often less (see figure 3.1.1). In contrast, slices cut in the coronal plane (methods, figure 2.1.1) consistently provided responses that were much larger and, moreover, appropriate for electrophysiological analysis. Therefore coronal sections were used in all subsequent experiments.

3.1.1.2 Slice cytoarchitecture

To visualise the cortical layers of the mouse perirhinal cortex and thus aid appropriate electrode placement, some slices were harvested from the electrophysiological chamber and stained using toluidine blue with an eosin pink counterstain (figure 3.1.2). As reported previously in other species, layer I was almost devoid of cell bodies, while layer II/III was characterised by the presence of pyramidal cells, the density of which was higher in layer II than layer III. Layer IV could be identified in area 36 (temporal cortex side), but was absent in area 35 (entorhinal cortex side). Within layer V were pyramidal cells that appear larger than cells of layers II/III, but less densely packed. Layer VI comprised of densely packed, polymorphic cell types.

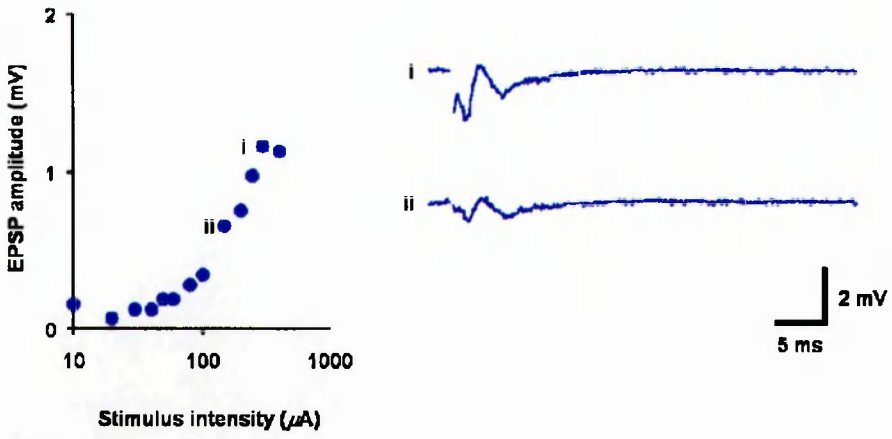


Figure 3.1.1 Field responses recorded from the intermediate pathways of an oblique slice. *Left* A typical input-output relationship. *Right* Typical field potentials (single traces) at the points indicated on the input-output relationship. Responses are clearly not suitable for electrophysiological analysis. Similar responses were obtained from the superficial pathways.

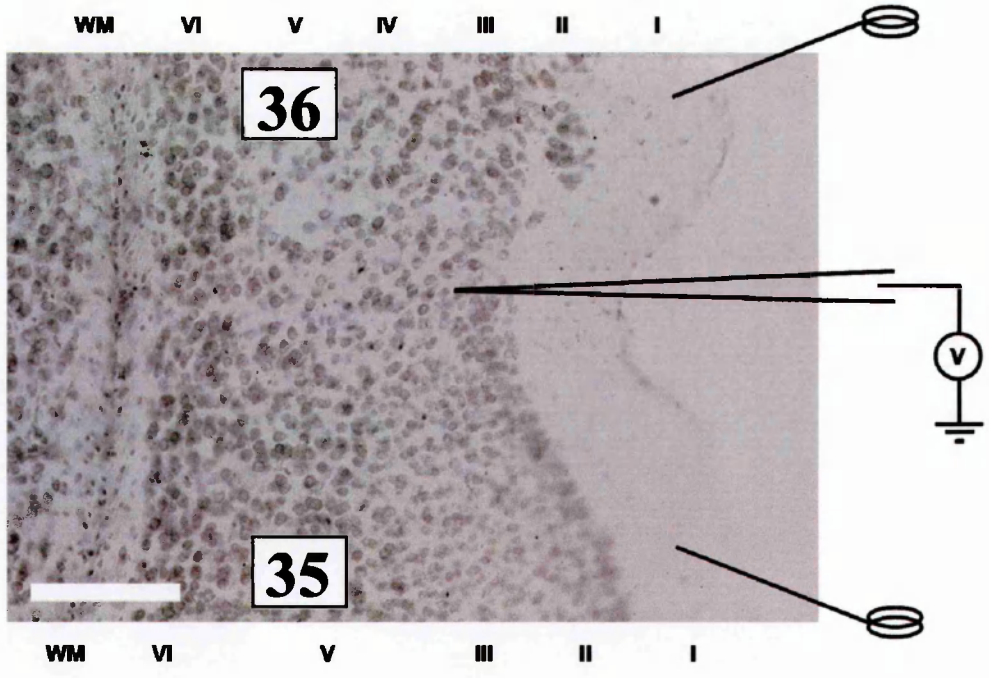


Figure 3.1.2 Slice architecture and electrode positioning. 50μm slices were stained using haematoxylin with an eosin counterstain. The typical perirhinal cortical structure can be seen, with the prominent rhinal sulcus to the right. Cortical layers are indicated above and below the slice. Layer I shows very few cells, while layer II is characterised by a relatively high density of pyramidal cells. Pyramidal neurones are more sparsely distributed within layer III. Layer IV is evident in Brodmann area 36, but not in area 35. Layer V contains larger pyramidal cells than layer II/III. Polymorphic cells are relatively densely packed in layer VI. The white mater is also evident. The positioning of electrodes is superimposed over the photograph. Scale bar 250 μm. Image obtained with a ×10 objective.

3.1.1.2 Intermediate pathways

Previous experiments in the rat perirhinal cortex were concentrated on the intermediate pathway. The intermediate pathway comprises predominantly of inputs from layers II/III of entorhinal and temporal cortices, which synapse onto basal dendrites of layer II/III pyramidal neurones of the perirhinal cortex. My initial experiments examined synaptic plasticity in this pathway.

Responses from the intermediate pathway were more difficult to obtain than by stimulation of the superficial layer, although responses that were recorded were suitable for analysis. A typical sigmoidal input-output relationship was obtained and is shown in figure 3.1.3 (maximum amplitude: 4.0±0.5 mV; n=18(6)).

Test-shock intensities were set to evoke responses at 30, 50 and 80% of the maximal amplitude. Pairs of stimuli were applied at intervals between 20 and 2000 ms. Unexpectedly, the intermediate pathways did not express paired-pulse depression (PPD), but favoured the expression of paired-pulse facilitation (PPF) (figure 3.1.3; n=36(11)). PPF was particularly evident between the intervals of 60 and 200 ms at both 30 and 50% stimulation intensities. A greater degree of facilitation was achieved at 30% than at 50% stimulation intensities. At 80% stimulation intensity the 20 ms interval gave rise to PPD. At intervals greater than 200 ms, the second response was the same as the first.

Long-term depression was not readily inducible in the intermediate pathways. Low-frequency stimulation led to a frequency dependent depression, however, of 13 slices, only three exceeded the criterion for LTD (greater than 10% depression at one hour) while another two came near to this value (>9% depression at one hour). At 30

minutes post-conditioning, the mean degree of depression was –9.6±4.0% and at 60 minutes was –6.7±2.8%. While these values do not meet the set criterion for LTD, they are both a significant degree of depression ($P<0.05$ at both time points; two-tailed, paired-t-test; n=13(8); figure 3.1.3).

3.1.2 Superficial synapses

While a significant LTD was induced at intermediate synapses, the incidence of LTD at these synapses is relatively low (23%), making pharmacological experiments designed to investigate LTD induction difficult. Furthermore, PPD does not appear to be supported at these synapses. Attention was therefore turned to the superficial synapses and these proved to be more plastic than those of the intermediate inputs and were therefore the target of extensive investigation as described below. This pathway comprises collateral inputs, predominantly from adjacent cortical regions, that synapse onto apical dendrites of layer II/III pyramidal cells.

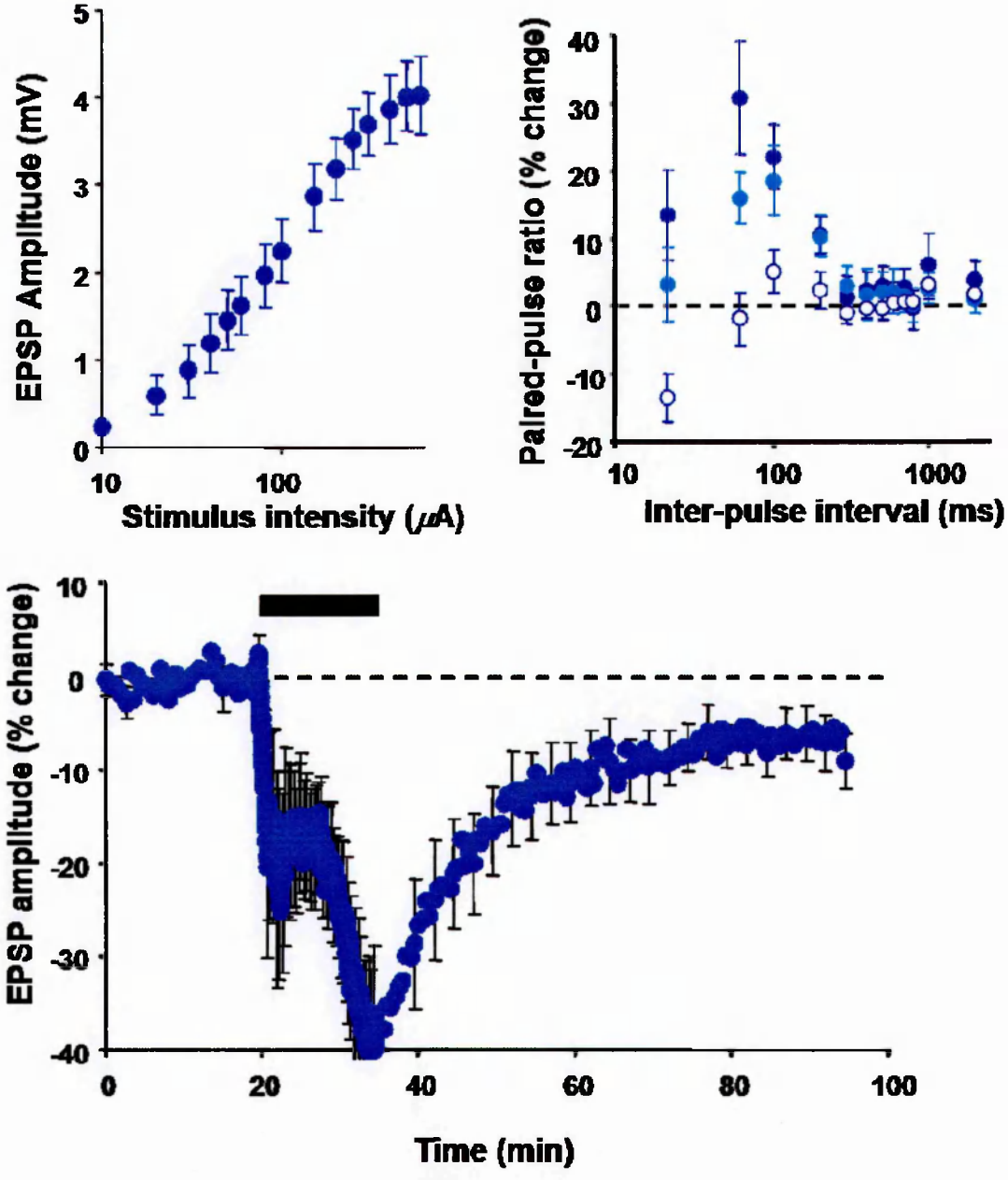


Figure 3.1.3 Plasticity in the intermediate pathway. *Top left* The intermediate pathway shows a typical input-output relationship in response to an increasing stimulus intensity (in this and subsequent input-output figures, the abscissa is plotted on a logarithmic scale). *Top right* Paired-pulse profiles obtained at 30 (•), 50 (•) and 80 (○) *per cent* maximal stimulation. Paired-pulse facilitation was seen at intervals up to 200 ms and no-change at intervals over 200 ms (in this and subsequent paired-pulse profile figures the abscissa is plotted on a logarithmic scale). *Bottom* Long-term depression in the intermediate pathway. The solid bar in this and subsequent figures represents the period of low-frequency stimulation; 900 pulses at 1 Hz.

3.1.2.1 Interpretation of the field response

In order to understand the cellular events underlying the recorded field response, dual recordings were made using both intracellular and extracellular recording techniques. Potentials were obtained simultaneously from layer II/III of the perirhinal cortex in response to increasing intensity test-shocks applied to layer I. Representative traces can be seen in figure 3.1.4. At stimulation intensities that evoke field responses greater than approximately 40% of the maximum amplitude, individual cells fire action potentials. Therefore, from ~40% maximum amplitude, the field potential is a mixed response comprising both an EPSP and population spike. Given that the recorded field response has a large population spike component, slope measurements are not meaningful and therefore amplitude was used as the measure of the field post-synaptic potential for all experiments in sections 3.1 and 3.2.

Bath application of the AMPA/KA receptor antagonist CNQX (20 μM) (Honore *et al.*, 1988) abolished the evoked response confirming the glutamatergic and synaptic origin of the recorded potentials (figure 3.1.5).

Increasing the stimulus intensity (10-600μA) increased the amplitude of the synaptic response and produced a typical sigmoidal input-output relationship (figure 3.1.5). There was no significant difference between the superficial temporal or entorhinal pathway assessed either by maximum amplitude (2.7±0.2 mV, n=27(16) and 2.9±0.2 mV, n=28(15), respectively; P>0.5, unpaired, two-tailed t-test) or as an overall profile (P>0.5, two-way ANOVA) and therefore data form these pathways were pooled (maximum amplitude: 2.8±0.2 mV, n=55 (21); figure 3.1.5).

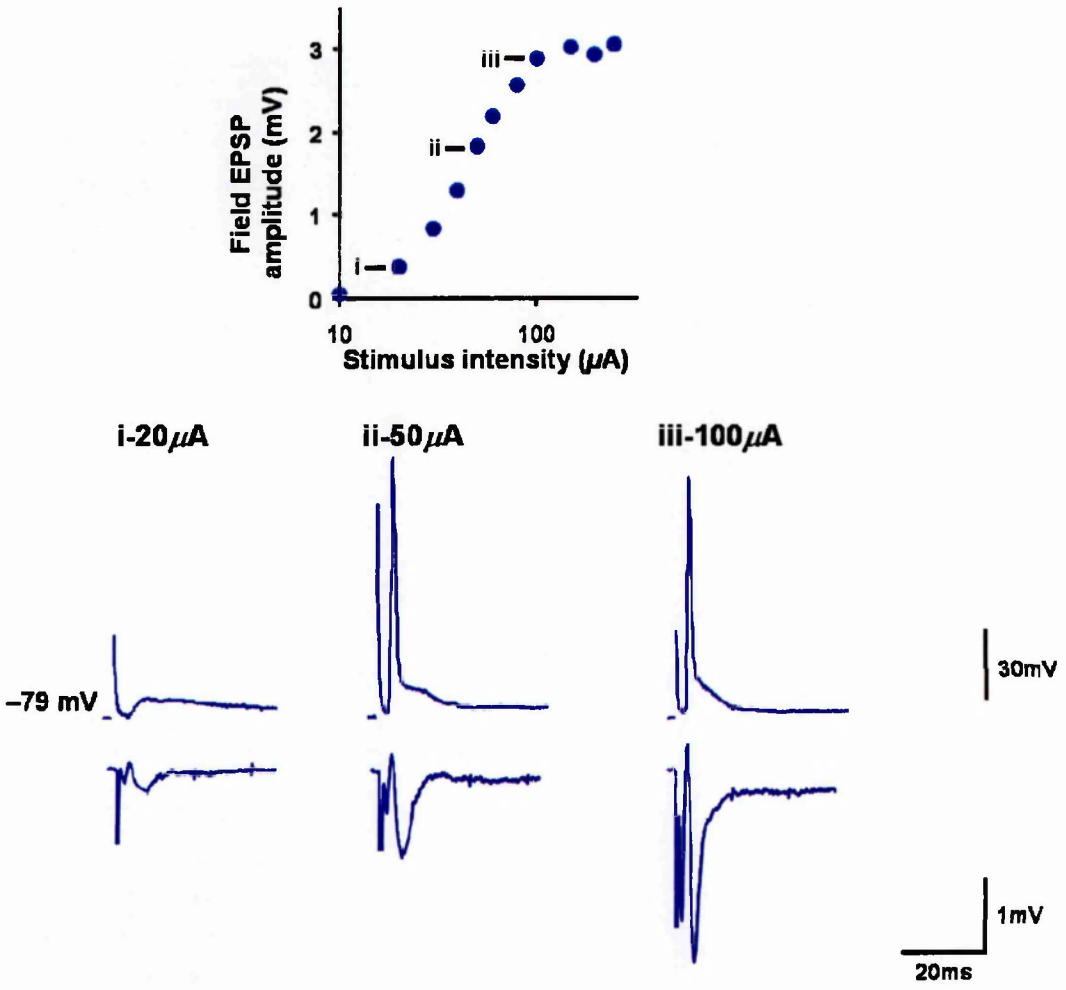


Figure 3.1.4 Interpretation of the field response. Dual intracellular and extracellular responses were made from layer II/III in response to increasing stimulus intensities. *Top* The synaptic response in this slice showed a typical sigmoidal input-output relationship. *Bottom* Responses recorded simultaneously *via* a sharp intracellular electrode (top row) and a field electrode (bottom row) in response to 20, 50 and 100 μA stimuli (corresponding approximately to 30, 50 and 80% stimulation intensities). It is apparent that the field response is dominated by a population spike at 50 and 100 μA stimuli. With the exception of responses that show an action potential, all traces are the average of five consecutive responses. Note the different vertical scale bar for intracellular and extracellular responses. All recordings were made in the absence of PTX

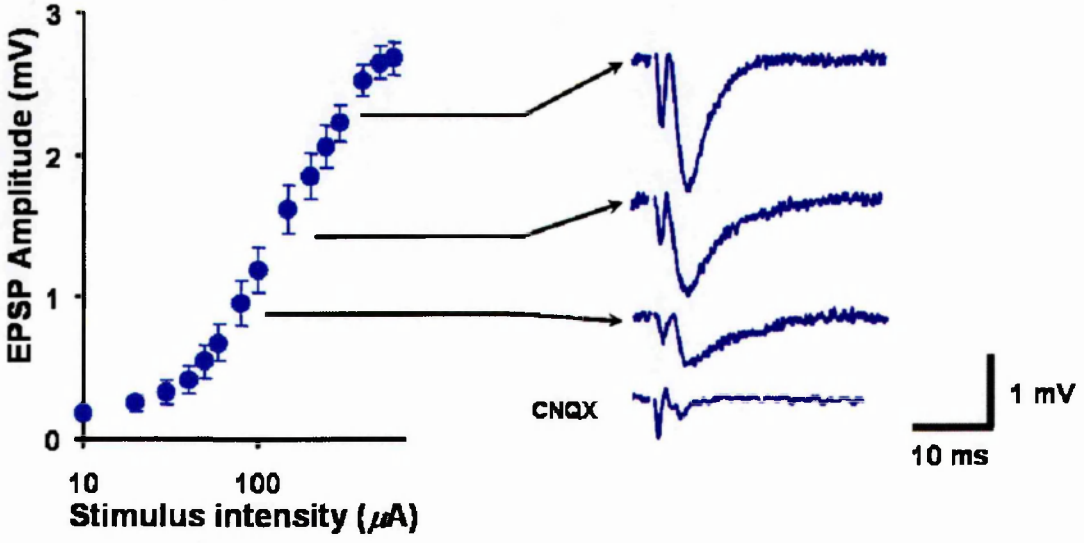


Figure 3.1.5 Input-output relationship in the superficial pathway. *Left* Typical sigmoidal input-output relationship (n= 55(19)). *Right* Single traces at 30, 50 and 80% stimulation intensities. The bottom trace shows the effect of the AMPA/KA receptor antagonist CNQX (10 μM), which abolishes the synaptic response.

3.1.2.2 Short-term plasticity

Short-term plasticity was examined at shock intensities set to evoke field responses at 30, 50 and 80% of the maximum amplitude (figure 3.1.5). Paired-pulse stimuli were applied at intervals between 20 and 2000 ms to the superficial layer of the cortex. At each of the three intensities, both the ST and SE pathways preferentially displayed PPD. No significant difference was found between the paired-pulse profiles exhibited by these pathways ($P>0.5$, two-way ANOVA) and therefore data from both pathways has been pooled (figure 3.1.6). PPD was found to be maximal at an inter-pulse interval of 20 ms and also between 400-600 ms. At 30 and 50%, a small degree of PPF was observed at inter-pulse intervals between 60 and 100 ms, whereas at 80% PPD was seen at all inter-pulse intervals up to and including 1000 ms. By increasing the stimulus intensity though 30, 50 and 80%, the degree of paired-pulse depression was increased for any given interval (figure 3.1.6).

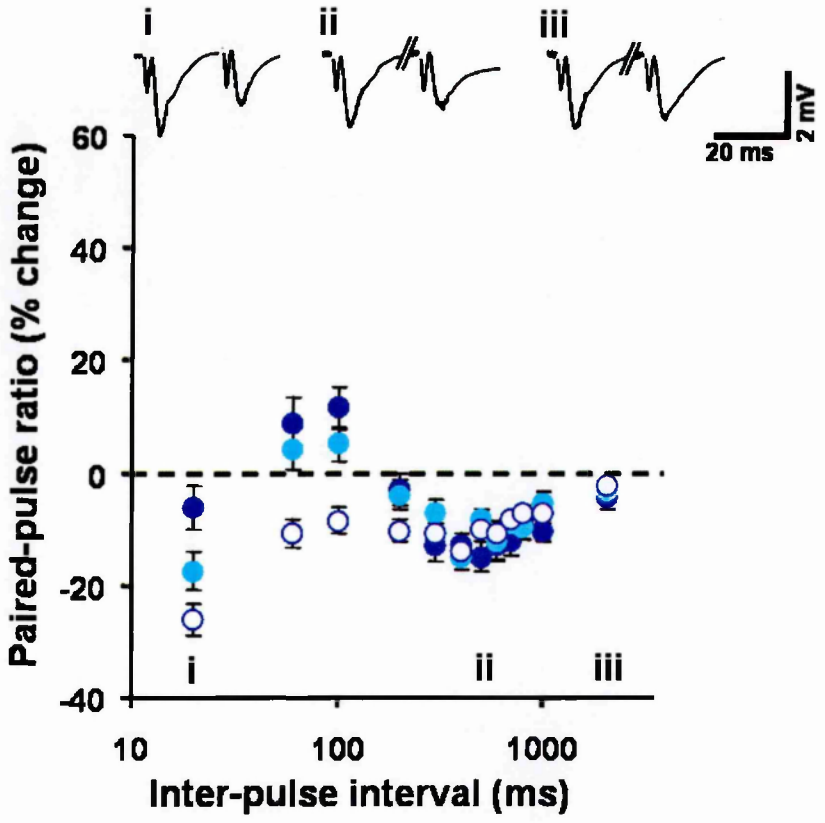


Figure 3.1.6 Paired-pulse depression in the superficial pathway. Paired pulses applied to the superficial pathway leads to depression of the second response at 30 (●), 50 (●) and 80% (○) stimulation intensities. At 30 and 50% paired-pulse facilitation is seen at intervals between 60 and 100 ms. Increasing the intensity though 30, 50 and 80% increases the degree of depression observed. n=48(15) for all three intensities. Example traces, obtained at 80% stimulation intensity, are shown from the time points indicated (i-iii) (averages of 5 consecutive traces).

3.1.2.3 Pathway independence

A paired-pulse protocol was used to assess pathway independence. Test-shocks were applied to alternate pathways at 60 ms intervals. Pathway independence was determined by the absence of heterosynaptic depression, while displaying homosynaptic depression. Further evidence for input specificity was provided by the coincidental application of a test-shock to both pathways leading to summation of the EPSP to amplitudes greater than the maximum recorded from either pathway alone (see figure 3.1.7 for an example). This analysis was carried out for all two-pathway analyses prior to experimentation and electrode placement altered to ensure pathway independence.

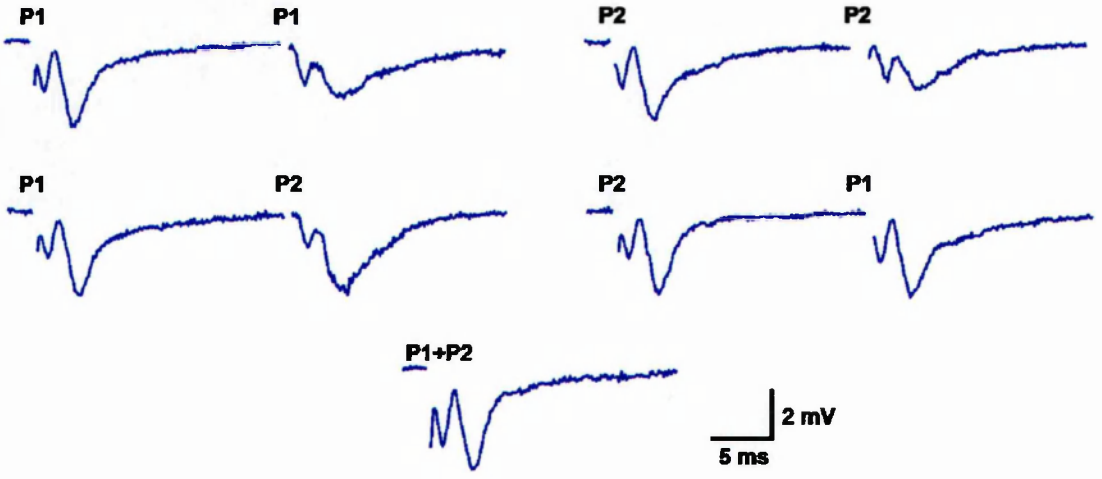


Figure 3.1.7: Determination of pathway independence. A paired-pulse protocol was used to ensure pathway independence. *Top row* Expression of homosynaptic PPD was ensured in each pathway by applying paired stimuli to an individual pathway (P1 or P2) at a 100 ms interval. *Middle row* The absence of heterosynaptic PPD was ensured by applying stimuli to the second pathway 100 ms after the first and *vice versa* . *Bottom* Summation of the EPSP occurred when both pathways were simultaneously stimulated, indicated a different population of synapses underlies the recorded EPSP in each pathway.

3.1.2.4 Long-term depression

A standard low-frequency conditioning paradigm (900 pulses applied at 1 Hz at test-shock intensity) was used in an attempt to induce LTD at superficial perirhinal synapses. A stable baseline was recorded for at least 15 minutes before a conditioning stimulus was applied. During the conditioning period a frequency-dependent depression was observed, characterised by a rapid decrease in EPSP amplitude during the first five minutes of conditioning. Following LFS a stable depression was observed for more than one hour. There was no significant difference between the levels of LTD expressed in the ST and SE pathways (−12.7±5.8%, n=8(5) and −13.3±5.0%, n=10(6), respectively; $P>0.5$, two-tailed unpaired t-test) and therefore data from both pathways has been pooled throughout this study (pooled LTD: −12.3±3.0%, n=20(9); figure 3.1.8; pooled LTD value obtained from all slices, irrespective of experimental outcome). LTD of greater than 10% was expressed in 60% of slices studied.

To determine whether LTD was homosynaptic, in some experiments a second pathway, which did not receive conditioning stimuli, was also monitored. For this analysis, only those pathways that exhibited LTD that was greater than 10% were included (in all other experiments throughout this thesis, data for each condition is pooled irrespective of outcome). Figure 3.1.8 demonstrates the homosynaptic nature of perirhinal LTD (conditioned pathway: −21.5±5.6%; unconditioned pathway +1.9±1.0%; n=5(5); $P<0.01$, paired, two-tailed t-test).

Induction paradigms

Alternative induction paradigms were investigated in an attempt to more reliably induce LTD. Paired-pulse low-frequency stimulation (two pulses with an inter-pulse interval of 60 or 100 ms, repeated 900 times at 1 Hz) did not increase the probability of LTD induction, or increase the degree of depression (summarised in table 3.1.1).

The $GABA_A$ antagonist PTX (100 μM) was added to ACSF in an attempt to aid LTD induction. PTX led to a large, late-onset component to the field EPSP and repeated stimulation at test-shock frequency (0.033 Hz) resulted in a decline in the EPSP until no response was recordable. This is likely to be a result of disinhibition of the cortical slice as, when 100 μM PTX was applied during whole cell recording, periods of prolonged spontaneous activity was evident. Reducing the PTX concentration to 2.5 μM prolonged the duration of the fast EPSP component, but did not lead to a late component. Quite the opposite to enhancing LTD, PTX prevented the induction of LTD induction and was not, therefore, utilised in field experiments. A similar result was found with the $GABA_A$ antagonist bicuculline methiodide (100 μM).

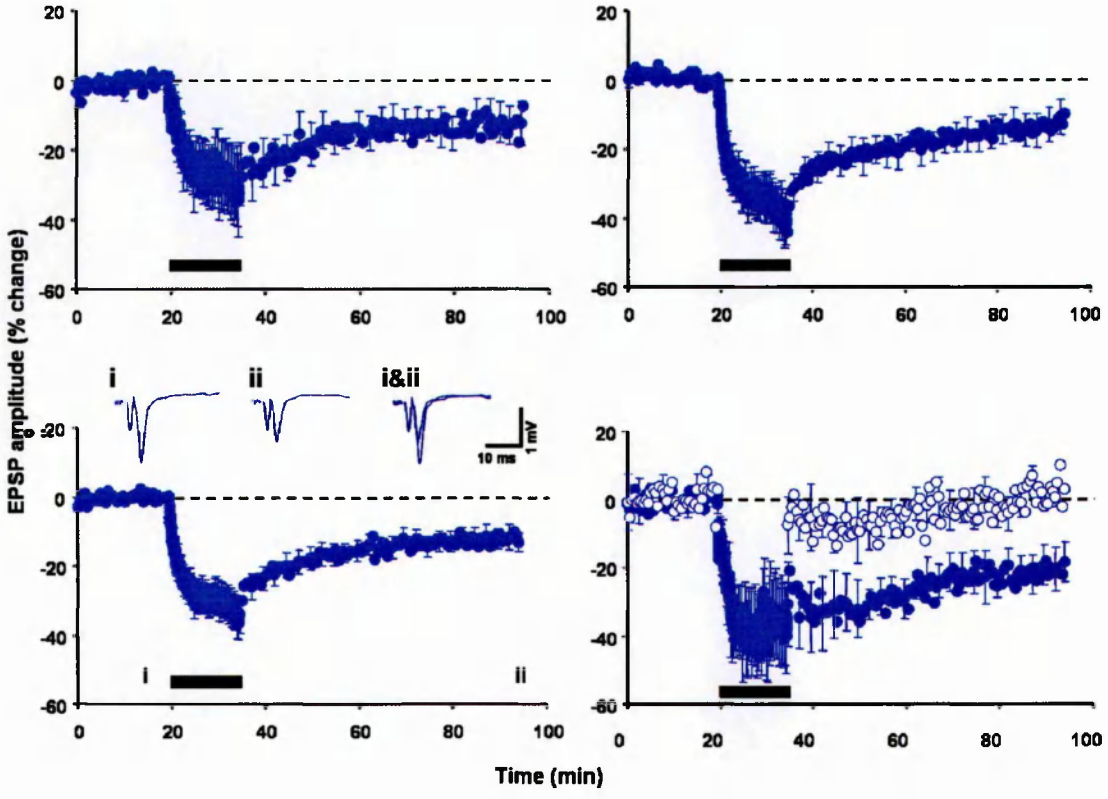


Figure 3.1.8 Homosynaptic LTD at superficial synapses. *Top left* Long-term depression in the superficial temporal pathway. *Top right* Long-term depression in the superficial entorhinal pathway. *Bottom left* No significant difference was found between the two pathways and were therefore pooled in all subsequent experiments. Typical traces are shown from baseline (i) and 60 minutes post-conditioning (ii) and are overlaid on the right (averages of 5 consecutive traces). *Bottom right* In some experiments two pathways were recorded, one of which did not receive LFS (○). Only the conditioned pathway (●) showed LTD. (For this analysis only slices showing LTD of greater than 10% were included, in all other experiments all data were included irrespective of outcome.)

Conditioning paradigm	Total trials (n)	LTD (n)	No change (n)	LTP (n)
900 pulses @ 1 Hz	20	12	8	0
900 paired-pulses (60 ms) @ 1 Hz	5	1	3	1
900 paired-pulses (100 ms) @ 1 Hz	7	4	3	0
2.5 μM PTX; 900 pulses @ 1 Hz	10	1	5	4
100μM BMI; 900 pulses @ 1 Hz	10	0	7	3

Table 3.1.1 Experimental outcomes of the paradigms used to induce LTD at superficial synapses of the mouse perirhinal cortex. Criteria for LTD or LTP were −10% and +10% respectively.

3.1.2.5 Long-term potentiation

High-frequency stimulation was used to induce long-term potentiation at superficial synapses in the mouse perirhinal cortex. Various tetanic stimulation paradigms were used in attempts to induce LTP (see table 3.1.2), none of which successfully induced LTP. Theta-burst stimulation, on the other hand, did induce reliable LTP. The optimum paradigm was four trains applied every 15s, each train comprising of 10 bursts of 10 pulses at 100 Hz, with an inter-burst interval of 100 ms.

No significant difference was found between the temporal and entorhinal pathways (16.5±5.8%, n=5(3) and 20.0±7.4%, n=6(4) respectively; $P>0.5$) and therefore data from the two pathways were pooled. TBS induced a potentiation (18.4 ± 4.7%; n=11(6); $P<0.01$; figure 3.1.9) that lasted for more than one hour after conditioning. Some experiments were designed to assess the homosynaptic nature of LTP. Only those experiments that showed greater than 10% potentiation were included in this analysis (in all other conditions, all experiments were included irrespective of outcome). The control pathway, which did not receive a conditioning stimulus, did not show potentiation (4.0±3.5%) and was significantly different than the experimental pathway (30.4±2.6%, n=5(3); $P<0.004$, paired-test; figure 3.1.9).

Conditioning paradigm	Total trials (n)	LTP (n)	No change (n)	LTD (n)
Tetanus: (1s 100 Hz)×4 every 15s	5	0	5	0
Tetanus: (1s 100 Hz)×6 every 15s	2	0	2	0
TBS: ((10@100 Hz)×10@5 Hz)×4 (15s)	9	4	5	0
TBS: ((10@100 Hz)×10@10 Hz)×4 (15s)	15	11	4	0
TBS in PTX (2.5μM)	5	0	3	2

Table 3.1.2 Outcomes of high-frequency paradigms used to induce LTP. Either tetanus (4 or 6 trains delivered every 15 s, each train 100 Hz, 1 s) or TBS (4 trains every 15s, each train comprising 10 bursts of 10 pulses at 100 Hz, inter-burst interval of either 100 or 200 ms). LTP or LTD was defined as +10 or –10% of baseline respectively.

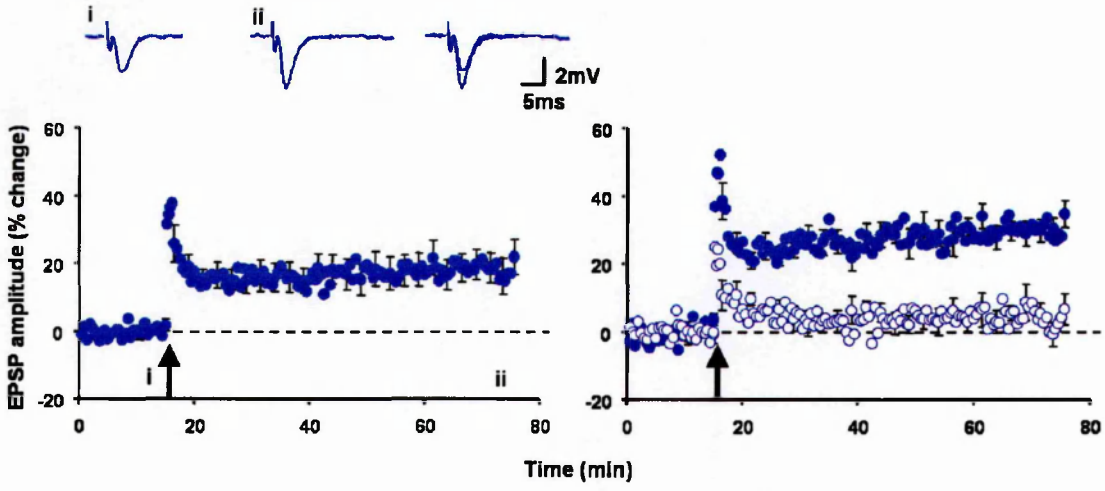


Figure 3.1.9 Homosynaptic LTP in the perirhinal cortex. *Left* Theta-burst stimulation (in this and subsequent figures TBS is represented by a solid arrow) induces a long-lasting potentiation at superficial synapses. Example traces are shown above from the time points indicated (i & ii) and are overlaid on the right (averages of 5 consecutive traces). *Right* In some experiments two pathways were recorded, one of which received TBS conditioning (•), the other did not (○). LTP was induced only in the conditioned pathway, indicating the homosynaptic nature of LTP in the perirhinal cortex of mice.

3.1.3 Intracellular recordings

Cellular properties of perirhinal neurones were assessed using sharp electrodes. Pyramidal neurones of layer II/III were impaled by stepping through the slice until a cell was found and then a small amount of negative current was applied to aid the formation of a seal between the cell membrane and glass electrode. Once this current was fully removed and the cell had stabilised, the electrophysiological properties were ascertained

3.1.3.1 Cellular properties

The properties of layer II/III perirhinal cortical neurones are summarised in table 3.1.3. Resting membrane potential (E_m) varied from approximately –66 mV to –90 mV with a mean of –79.2±0.6 mV (n= 51 cells). The distribution of E_m followed a Gaussian distribution (figure 3.1.10; P>0.3). Injection of square, negative current pulses (–0.2 to –0.8 nA) into the cell gave rise to a hyperpolarisation (figure 3.1.10). Initially following the current injection, a charging of the cell membrane was observed until a steady-state membrane potential was reached. The slope of the plot of current injection against voltage deflection at steady-state was used to calculate the membrane input resistance (R_{in}). The R^2 values for the best-fit lines were greater than 0.98 and gave a mean R_{in} of 39.2±2.4 MΩ (n=35 cells). Membrane capacitance (C_m) was calculated from the membrane input resistance and the time constant (τ_m) using the following relationship: $C_m = \tau_m / R_{in}$. Values of τ_m were obtained using the

protocol in Bindman *et al.* (1988) as follows: Semi-logarithmic plots were made of the voltage response against time during the charging of the cell by hyperpolarising current pulses. The charging response was always well fitted by a single exponential line (R^2 value greater than 0.99) and the time constant calculated as the negative reciprocal of the best-fit line at $(1-1/e \times 100)\%$ of the maximum deflection. τ_m was found to be 9.1±0.7 ms. Membrane capacitance was then calculated for each cell and was found to be 0.24±0.02 nF.

Injection of positive current (+0.2 to +2.0 nA) gave rise to a depolarisation. Action potentials were evoked at depolarised membrane potentials above the action potential threshold (–51.3±1.3 mV). The action potential typically overshot to +26.8±2.7 mV, with a mean action potential amplitude of 78.2±1.8 mV. The action potential duration (measured at half way between threshold and peak) was 1.83±0.09 ms (n=35 cells). All the cells impaled in this study were of regular-spiking characteristics; no late-spiking neurones, as described by Beggs *et al.* (2000), were identified. Cells typically fired within the first 500 ms of a depolarising current step and showed rapid accommodation, settling to a firing rate of approximately 1 Hz (for example, see figure 3.1.10).

When an EPSP was evoked during a sub-threshold depolarising current step, a substantial inhibitory component was evident. The $GABA_A$ receptor antagonist PTX was therefore bath applied. 100 μM PTX resulted in epileptiform activity and excessive spontaneous activity. The relatively low concentration of 2.5 μM PTX abolished the inhibitory component of the post-synaptic potential, while not inducing inappropriate activity. 2.5μM PTX was therefore used in all intracellular experiments to ensure a pure EPSP.

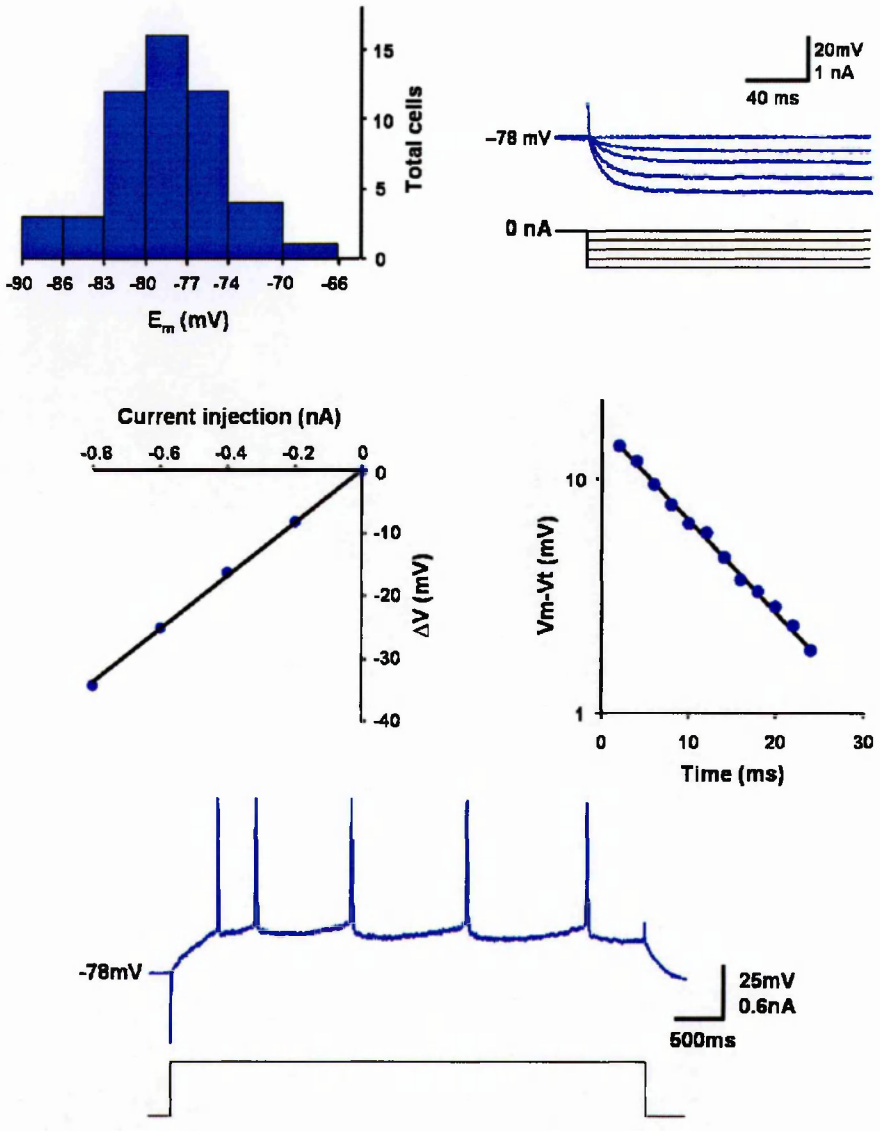


Figure 3.1.10 Passive membrane properties of layer II/III perirhinal cortical cells. *Top left* Resting membrane potential follows a normal Gaussian distribution. *Top right* Membrane deflections in response to hyperpolarising (0, −0.2, −0.4, −0.6 and −0.8 nA) square current pulses. Following an initial charging of the membrane, a steady state membrane potential is reached (averages of 4 consecutive traces). *Middle left* Current-voltage plot of the traces shown top left. The slope of the best-fit line (R^2>0.99) equals the input resistance (R_{in}). *Middle right* Time course of the voltage deflection obtained in response to −0.6 nA current injection. The difference of the maximum deflection (V_m) and the voltage at a given time after current injection (V_t) is plotted on a logarithmic scale. The negative reciprocal of the exponential line of best fit (R^2>0.99) was used to calculate the membrane time constant τ_m. *Bottom* Response to a positive current injection (+0.6 nA). Action potentials fire at membrane potentials above the threshold of −52 mV. The cell showed rapid accommodation, settling to a firing rate of approximately 1 Hz.

Cellular property	Mean ± SEM	n
E_m	−79.2 ± 0.6 mV	51
R_{in}	39.2 ± 2.4 MΩ	35
τ_m	9.1 ± 0.7 ms	
C_m	0.24 ± 0.02 nF	
$AP_{threshold}$	−51.3 ± 1.3 mV	
$AP_{overshoot}$	+26.8 ± 2.7 mV	
$AP_{amplitude}$	78.2 ± 1.8 mV	
$AP_{width\ at\ half\ amplitude}$	1.83 ± 0.09 ms	

Table 3.1.3 Electrophysiological properties of neurones within layer II/III of the perirhinal cortex. Intracellular recordings were made from cells within layer II/III of the perirhinal cortex. The current-voltage relationship was used to calculate input resistance, membrane time constant and, from these values, the capacitance of the cell. Action potential (AP) amplitude was calculated from threshold to maximum overshoot. Action potential width was measured at half amplitude.

3.1.3.2 Short-term plasticity

Paired-pulse stimuli were applied to the superficial layer whilst recording intracellularly from layer II/III neurones. Test-shock intensity was set to evoke responses at 50% of the action potential threshold value. At paired-pulse intervals up to 200 ms the second response was larger than the first. At intervals over 200 ms, the second response was similar in size to the first response. Typical responses and the mean paired-pulse profile are shown in figure 3.1.11.

3.1.3.3 Intracellular LTD

Long-term depression was also studied using sharp intracellular electrodes. EPSP slope was set to 50% of the threshold value and a stable baseline recorded for at least 10 minutes. The standard LFS paradigm of 900 pulses at 1 Hz was not suitable for inducing LTD at mouse perirhinal synapses under these conditions; LFS invariably resulted in loss of the intracellular recording during the conditioning period.

In the rat studies carried out in the Bashir Laboratory (for example see Cho *et al.*, 2000), a shorter LFS paradigm (300 pulses at 1 Hz) has been used to induce LTD recorded at the intracellular level. This paradigm did not compromise the integrity of the recording when applied to mouse perirhinal afferents. During the conditioning paradigm there was a frequency-dependent depression, however, subsequent responses were at baseline values (EPSP slope: –0.1±3.3%; n=8(6); figure 3.1.12). Pairing conditioning with depolarising current such that the cell spiked during each conditioning stimulus did not aid LTD induction.

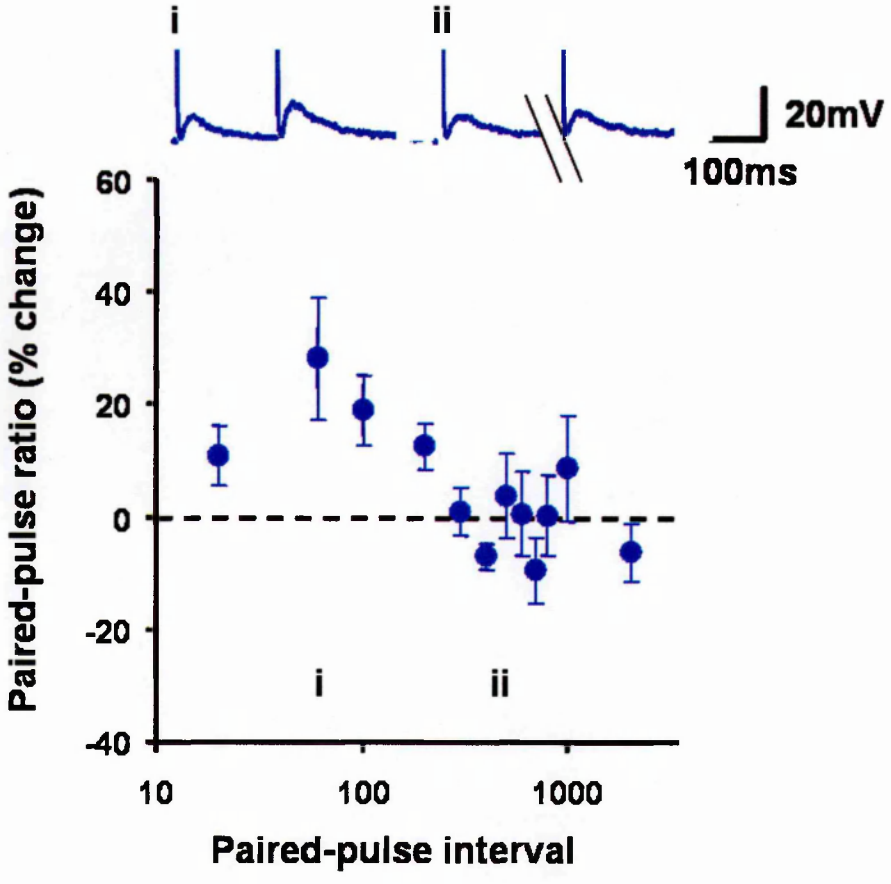


Figure 3.1.11 Intracellularly recorded paired-pulse profiles. Paired-pulse stimuli applied to layer I led to paired-pulse profiles favouring PPF. Example traces are shown above from 60 and 500 ms intervals (single traces).

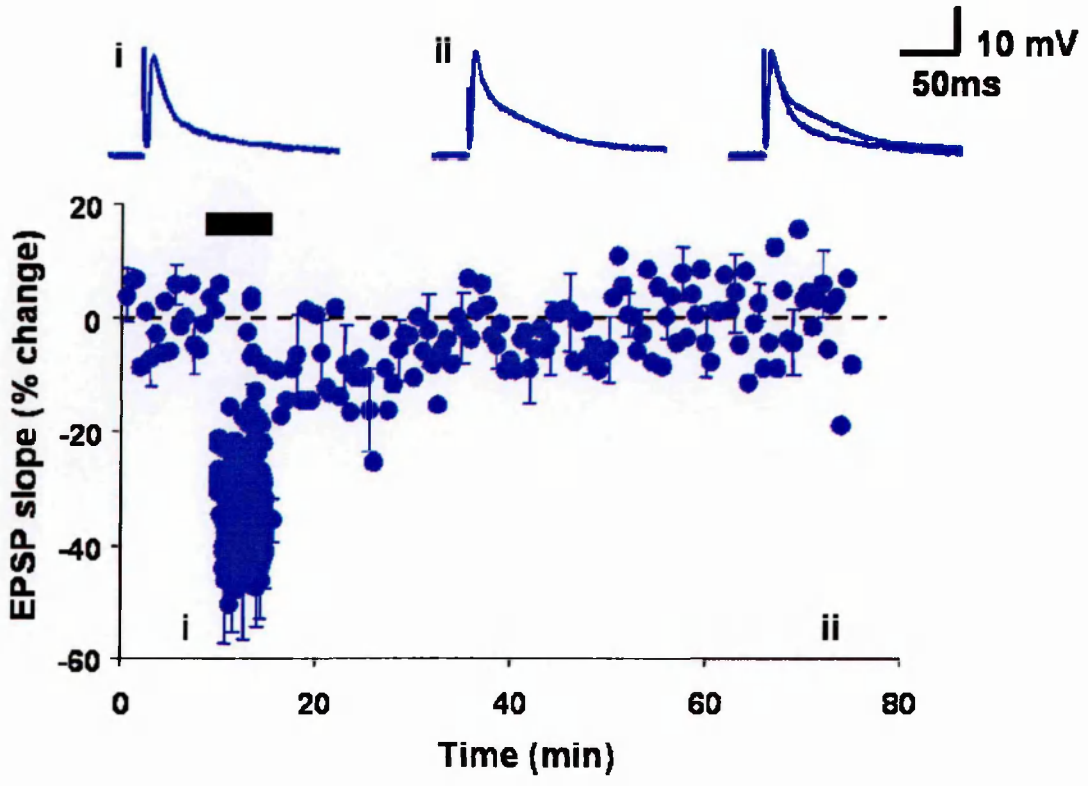


Figure 3.1.12 Aplasticity in the superficial pathways when recorded intracellularly. LFS (300 pulses at 1 Hz) failed to induce LTD in the superficial pathways. Typical responses are shown from the baseline (i) and 60 minutes post-conditioning (ii) and are overlaid on the right (average of 5 traces).

3.1.3.4 Spike LTD

In the absence of plasticity of the EPSP, the plasticity of the suprathreshold action potential was investigated. During baseline recordings responses were set to suprathreshold levels and, interestingly, LFS (300 pulses at 1 Hz) led to a long-lasting reduction in the number of test-shocks that evoked an action potential (spike-depression). During the baseline period, test-shocks evoked action potentials in >75% of trials. In contrast, following LFS, action potentials were evoked in <30% of post-conditioning trials (n=3(3)). Of the three experiments, two showed a complete absence of firing following conditioning, (figure 3.1.13). Evoked responses remained subthreshold for the duration of recording (up to one hour). An alternative pathway, which did not receive LFS, did not show spike-depression, but could be subsequently depressed upon application of LFS to this pathway. Importantly, in the absence of a conditioning stimulus, cells remained suprathreshold for the duration of the experiment (up to 7 hours; n=3(3); figure 3.1.14).

The induction of spike-depression was not a function of firing of the postsynaptic cell. 300 pulses (1 Hz) of depolarising current, sufficient to bring the neurone to firing threshold, did not lead to the induction of spike depression (n=3(3)) but subsequent LFS applied to superficial afferent inputs did lead to spike-depression (figure 3.1.15).

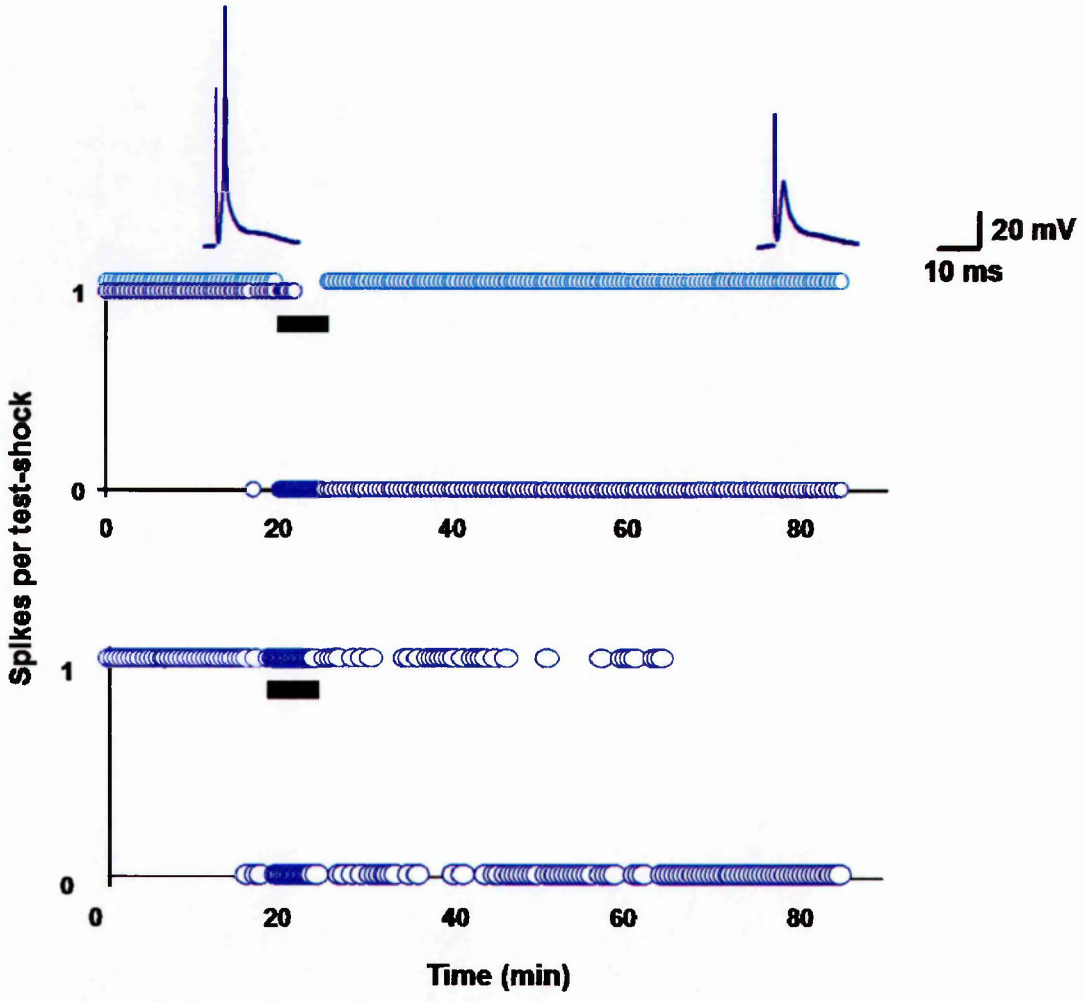


Figure 3.1.13 Long-term spike depression. *Top* Homosynaptic depression of action potential firing in the perirhinal cortex. Two independent pathways were stimulated at just suprathreshold intensities. One pathway received LFS (300 pulses at 1 Hz; conditioned pathway;○), the second pathway received no LFS (non-conditioned pathway; ○). Only the conditioned pathway showed spike depression. Example traces are shown above from the baseline and at 60 minutes post-conditioning. *Bottom* Another example of spike LTD demonstrated in a different slice.

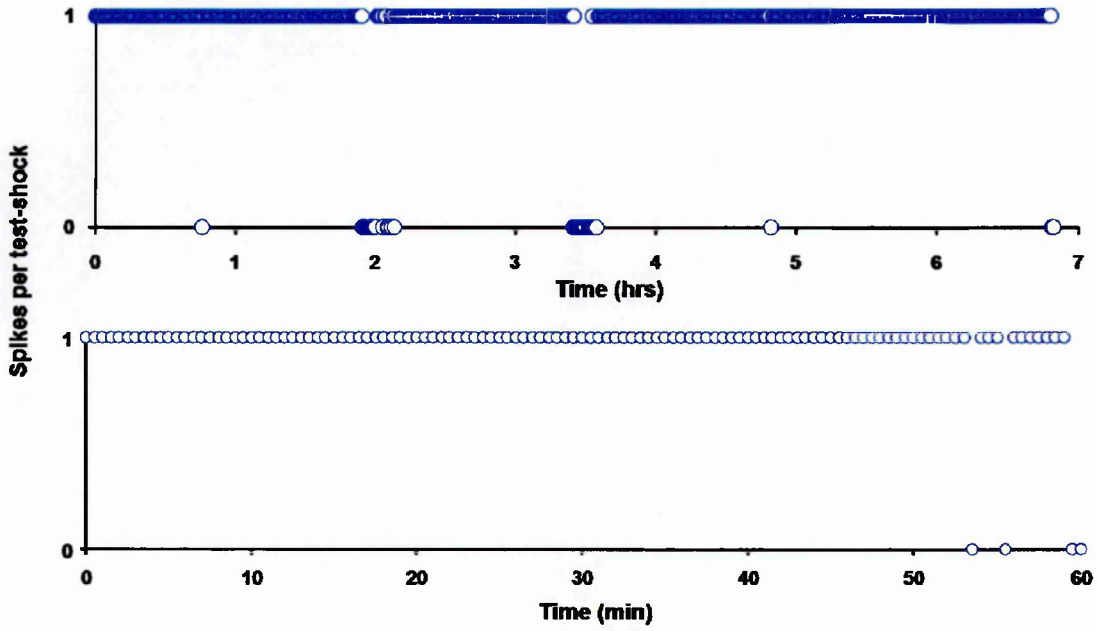


Figure 3.1.14 Persistent action potential firing in the absence of conditioning. Two examples demonstrating that, in the absence of conditioning stimuli, cells continue to fire for the duration of recording. Note the different time scales.

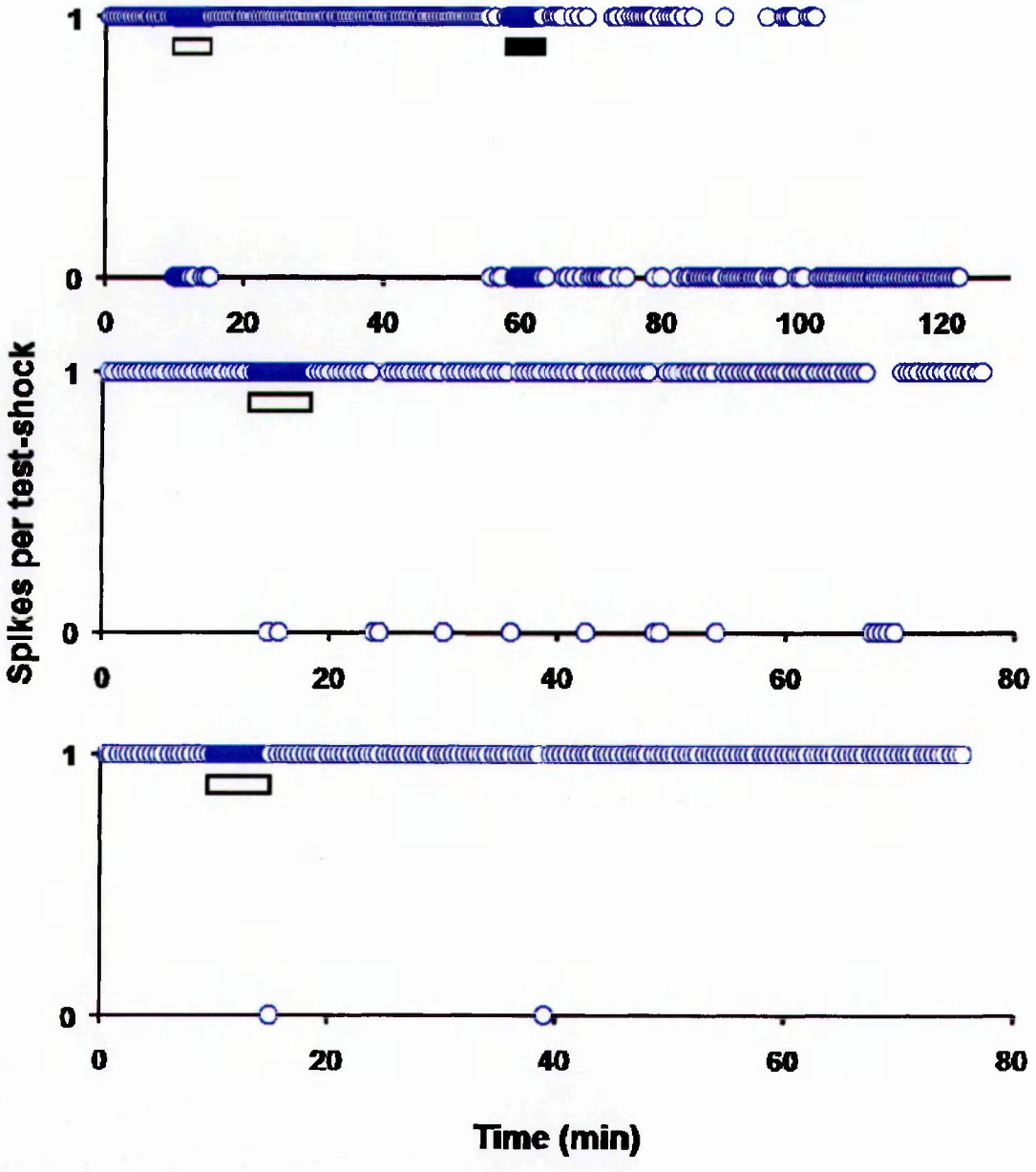


Figure 3.1.15 Spike LTD is dependent upon synaptic activity. *Top* Action potentials were evoked during baseline recording. The first period of LFS (open bar) comprised of 300 pulses of square depolarising current pulses such that action potentials were evoked. Following conditioning the cell continued to fire action potentials in response to test-shocks. A second period of LFS, this time consisting of synaptically evoked action potentials, resulted in an increase in the number of failures to evoke an action potential in response to test-shocks. *Middle and bottom* Further examples of cellular depolarisation by current injection failing to induce spike LTD.

3.1.4 Summary

Similarly to synapses of the rat perirhinal cortex, mouse perirhinal cortical synapses support paired-pulse depression, long-term depression and long-term potentiation. Cells within layer II/III are typical of other neocortical neurones, with a resting membrane potential at around –80 mV. While LTD of the intracellularly recorded EPSP was not observed, a long-lasting, homosynaptic depression of action potential firing, that was dependent on synaptic activation, could be induced by LFS applied to the superficial afferent pathways.

3.2 Neurotransmitter modulation of synaptic plasticity

Having identified some of the forms of synaptic plasticity expressed at mouse perirhinal synapses, the neurotransmitter subclasses involved in the induction of plasticity were investigated. Previously, LTD in the rat was found to be co-dependent on both NMDA and mGlu receptors (Cho *et al.*, 2000). The neurotransmitter receptor-dependency of synaptic plasticity in the mouse is summarised in figure 3.2.11.

3.2.1 Glutamate receptors

Initially the glutamate receptor subclasses were investigated using bath application of group-specific antagonists.

3.2.1.1 NMDA receptors

The involvement of NMDA receptors in mouse perirhinal synaptic plasticity was investigated at the field level. Bath application of the NMDA receptor antagonist, D-(−)-2-amino-5-phosphonopentanoic acid (D-AP5, 50μM) (Evans *et al.*, 1982) decreased the field potential. This is reflected in the input-output relationship, which, as a profile, is significantly different than control ($P=0.013$, $F_{14, 1080}=2.03$). The maximal amplitude (2.8±0.1 mV, n=14(4)) was not, however altered ($P>0.3$, two-tailed Welch test).

D-AP5 did not significantly alter paired-pulse depression ($P>0.3$; two-way ANOVA; see figure 3.2.1). Unexpectedly, D-AP5 did not block the induction of LTD (4 out of 10 experiments showed LTD), however, the pattern of transmission was altered. Initially, during the conditioning period the evoked response was dramatically reduced, but after the first five minutes of conditioning, the response tended to recover towards control levels. Immediately after conditioning, the EPSP amplitude returned to baseline values, but then decreased to give a stable LTD ($-10.4\pm5.9\%$; $n=10(4)$;$P<0.05$; paired t-test; figure 3.2.1) that was not significantly different than controls ($-12.3\pm3.0\%$, $n=20(9)$; $P>0.5$, two-tailed unpaired Welch-test). LTD obtained in the presence of D-AP5 is also homosynaptic (data not shown).

Similarly, D-AP5 did not effect the induction of LTP. Following TBS, a potentiation of $22.3 \pm 6.1\%$ ($n=8(4)$; $P<0.01$, paired t-test) was observed, which was not significantly different than control ($18.4 \pm 4.7\%$; $n=11(6)$; $P>0.5$, two-tailed unpaired Welch-test; figure 3.2.1).

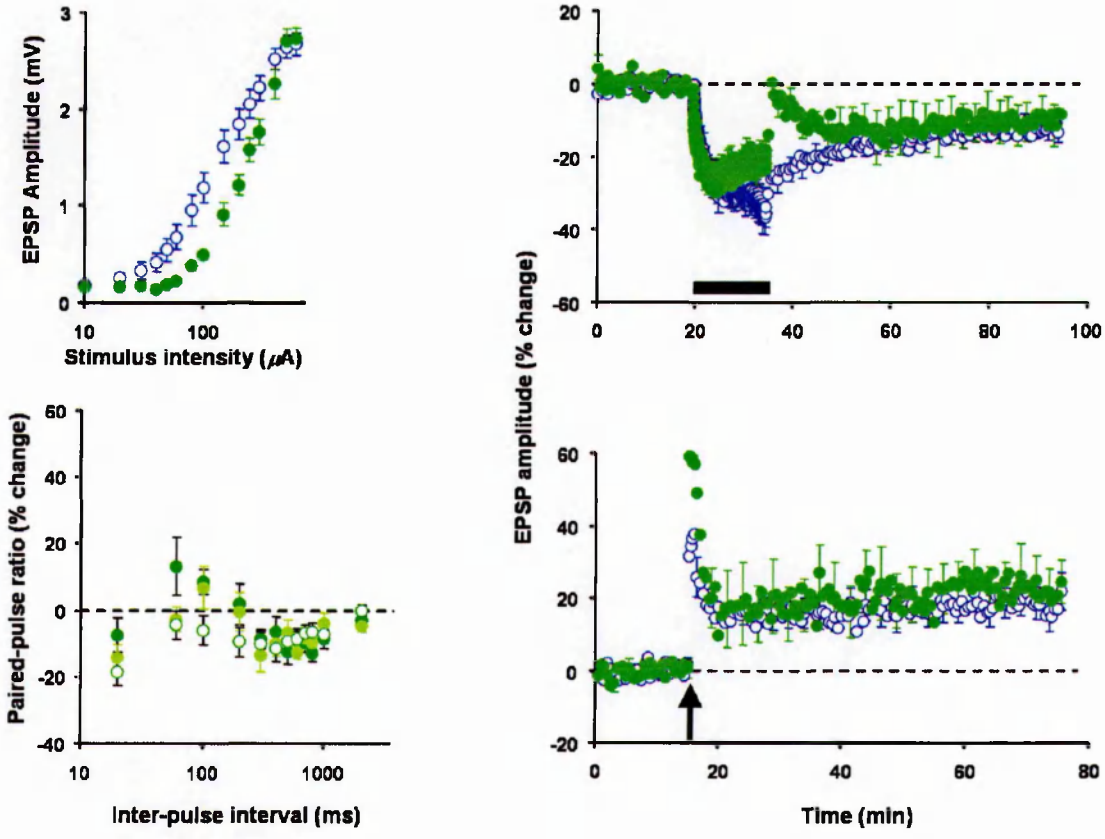


Figure 3.2.1 NMDA receptor-independent plasticity in the mouse perirhinal cortex. *Top left* 50 μM D-AP5 (in this and subsequent figures drug-conditions are represented by ●) altered the input-output curve, but did not alter the maximum amplitude compared to that of control experiments (this and subsequent figures: ○). *Bottom Left* Paired-pulse depression was not affected by the addition of D-AP5 at all three stimulation intensities (in this and subsequent drug-condition figures: 30(●), 50(○) and 80(○)%). *Top right* LTD was still induced in the presence of D-AP5. The drug did, however, alter the profile of the conditioning period and prevented post-conditioning depression. *Bottom right* LTP was unaffected by D-AP5.

3.2.1.2 Group I metabotropic receptors

The participation of metabotropic glutamate receptors in both paired-pulse plasticity and long-term depression was examined. Bath application of the group I mGlu receptor antagonist UPF 523/(RS)-1-aminoindan-1,5-dicarboxylic acid (AIDA, 500 μM) (Pellicciari *et al.*, 1995) did not alter basal transmission (see input-output relationship in figure 3.2.2).

AIDA abolished PPD at all stimulus intensities, producing paired-pulse profiles that favoured facilitation at both 30 and 50% stimulation intensities, but neither PPD nor PPF at 80% (figure 3.2.2). The change in profile was found to be statistically significant ($P<0.01$, $F_{11, 729}=2.30$ and $P<0.05$, $F_{11, 732}=1.76$ for 30 and 80% test shock intensities respectively; two-way ANOVA).

AIDA also affected the induction and expression of LTD. The degree of frequency-dependent depression seen during the conditioning period was reduced compared to control slices. Following conditioning, a transient depression was observed, but responses returned to baseline levels within ten minutes. Moreover, blocking group I mGlu receptors prevented the induction of LTD in all ten slices studied (2.6 ± 1.7%; n=10(6); figure 3.2.2). This result is significantly different than the LTD seen in control slices (–12.3±3.0%, n=20(9); $P<0.001$; unpaired, two-tailed Welch t-test).

Conversely, AIDA did not alter the degree of LTP induced by TBS (17.3 ± 8%; n=8(3)) compared to controls (18.4 ± 4.7%; n=11(6); $P>0.5$, two-tailed unpaired Welch-test; figure 3.2.2).

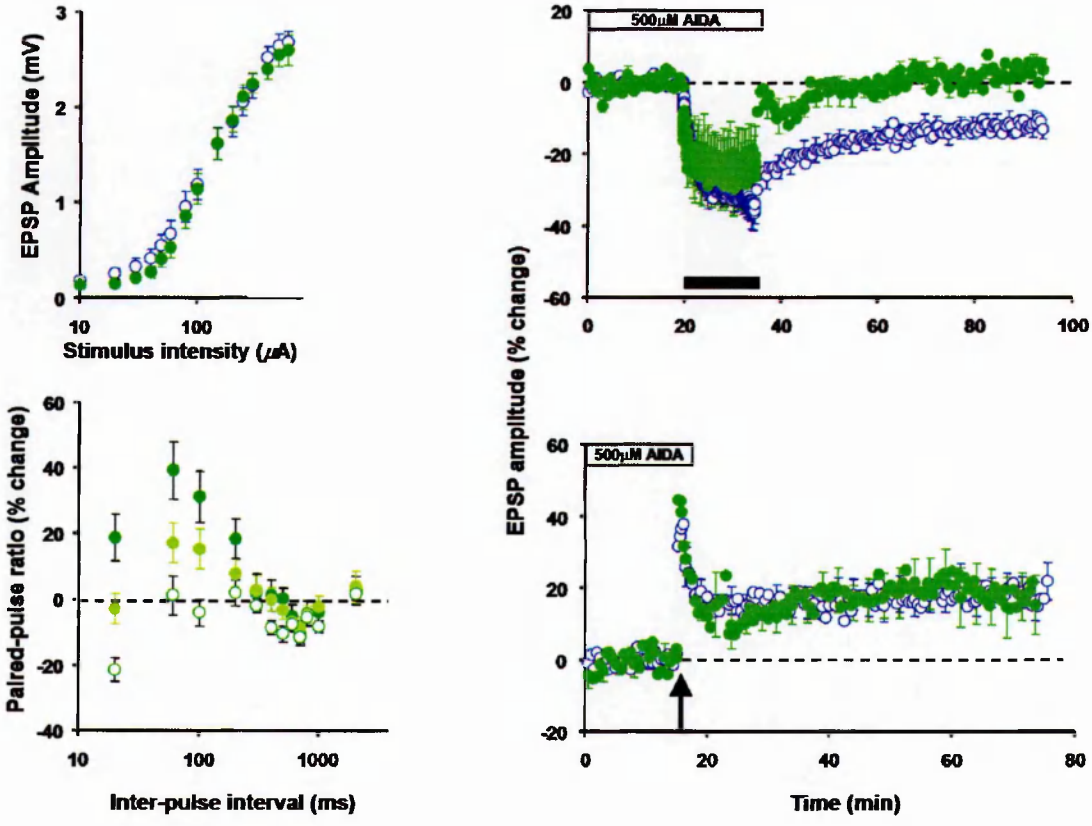


Figure 3.2.2 Group I mGlu receptor dependency of synaptic plasticity in the mouse perirhinal cortex. *Top left* The group I mGlu receptor antagonist AIDA did not alter the input-output relationship. *Bottom left* Antagonising group I mGlu receptors shifted the paired-pulse profile towards that of facilitation at all three stimulus intensities. *Top right* AIDA blocked the induction of LTD at superficial synapses, but did not affect LTP induction (*bottom right).* For fiscal reasons, AIDA was only present during the baseline and conditioning periods of the experiments investigating long-term synaptic plasticity.

3.2.1.3 Group II metabotropic receptors

The group II mGlu receptor-specific antagonist LY341495 (1 μM) (Kingston *et al.*, 1998) was applied to an additional set of slices. Drug application did not significantly alter input-output relationships compared to controls (P>0.5, two-way ANOVA; figure 3.2.3). When paired-pulse stimuli were applied in the presence of LY341495, facilitation was produced upon the second stimulus at all intensities in the paired-pulse paradigm (figure 3.2.3). The change in profile was significantly different than control values (P<0.01, $F_{11, 657} = 2.45$ and P<0.01, $F_{11, 648} = 2.60$ for 30 and 80% stimulus intensities respectively; two-way ANOVA).

LY341495 also suppressed the level of frequency-dependent depression seen during conditioning and, moreover, prevented the induction of LTD in 9 out of 10 experiments (−1.1 ± 2.7%; n=10(3); significantly different than controls; −12.3±3.0%, n=20(9); P<0.01, unpaired, two-tailed Welch t-test; figure 3.2.3).

Antagonising group II mGlu receptors did not alter LTP. There was no significant difference in the degree of potentiation at 1 hr after conditioning observed between control (18.4 ± 4.7%; n=11(6)) and drug conditions (20.9±4.3%; n=8(2); P>0.5; figure 3.2.3).

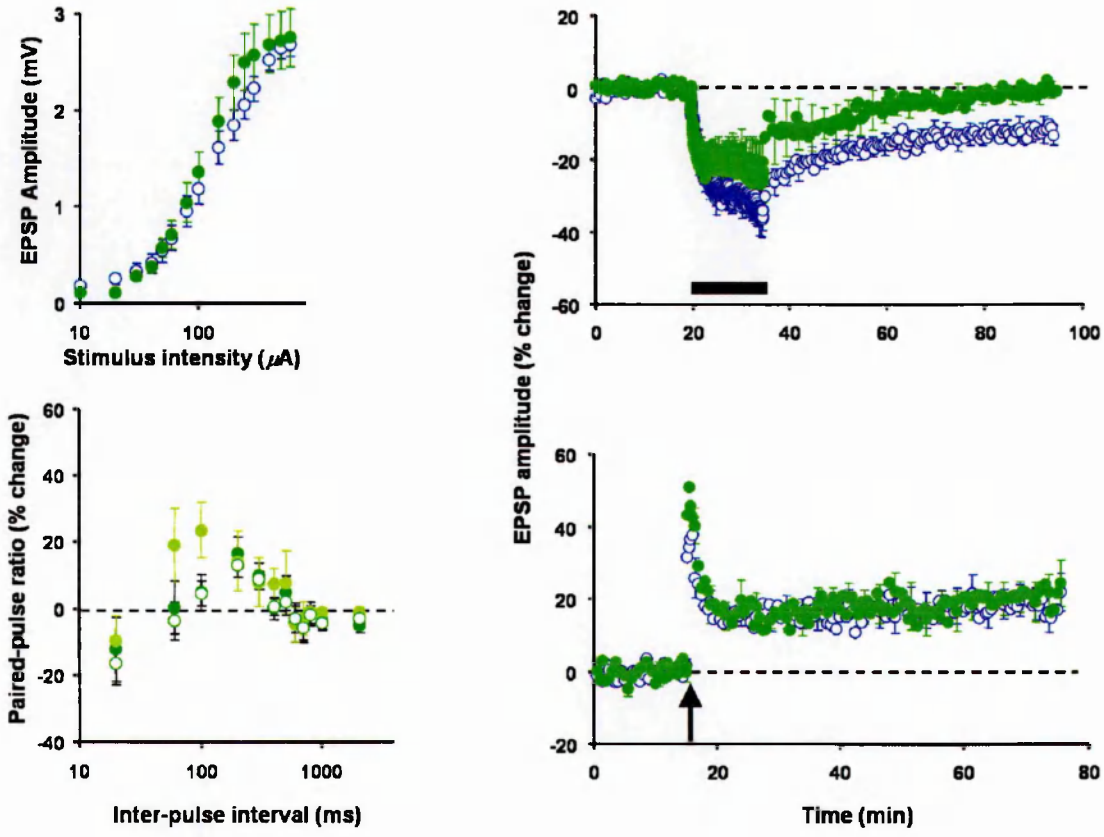


Figure 3.2.3 Group II mGlu receptor dependency of synaptic plasticity in the mouse perirhinal cortex. *Top left* The group II mGlu receptor antagonist LY341495 did not alter the input-output relationship. *Bottom left* Antagonising group II mGlu receptors shifted the paired-pulse profile to that of facilitation at all three stimulus intensities. *Top right* LY341495 blocked the induction of LTD at superficial synapses, but did not affect LTP induction *(bottom right).*

3.2.1.4 Group III metabotropic receptors

In contrast to the group I and II mGlu receptor-dependency, antagonism of group III mGlu receptors using 10 μM (RS)-α-Cyclopropyl-4-phosphonophenylglycine (CPPG) (Jane *et al.*, 1996; Toms *et al.*, 1996) did affect the overall input-output relationship ($P<0.001$, $F_{14, 815}=15.07$, two-way ANOVA) and increased the maximal amplitude to 3.3±0.2 mV (n=10(4); $P<0.01$, two-tailed t-test versus control). Also in contrast to antagonism of groups I and II mGlu receptors, CPPG did not affect paired-pulse depression ($P>0.5$, two-way ANOVA; figure 3.2.4) or the induction of LTD (−11.2 ± 3.1%), which is not significantly different than controls (−12.3±3.0%, n=20(9); $P>0.5$; two-tailed, unpaired t-test; n=6(3); figure 3.2.4). In the presence of CPPG, there was a trend for TBS to evoke a smaller degree of potentiation (10.1±7.82; n=8(3)) than in control experiments (18.4 ± 4.7%; n=11(6); figure 3.2.4), however this failed to reach significance ($P>0.3$; two-tailed, unpaired Welch-test).

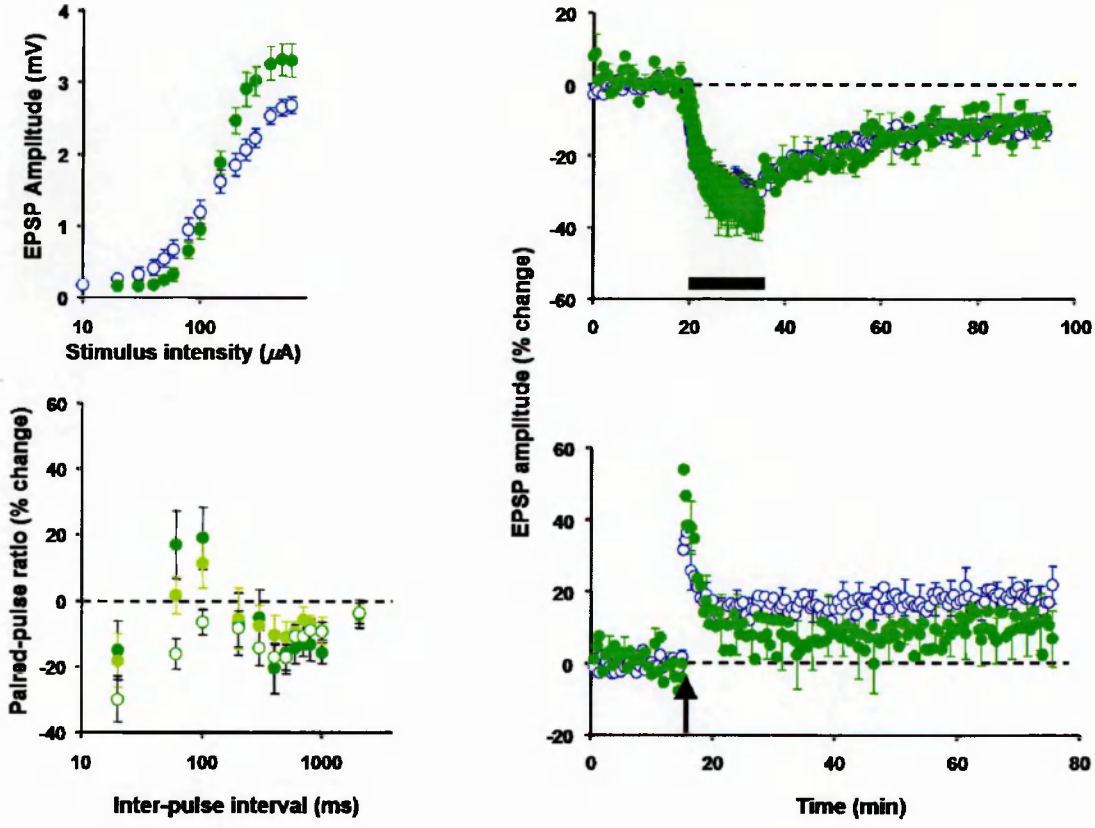


Figure 3.2.4 Group III mGlu receptor dependency of synaptic plasticity in the mouse perirhinal cortex. While the group III mGlu receptor antagonist CPPG altered the input-output relationship (*top left*) paired-pulse ratios (*bottom left*) were not affected by the drug. Nor did CPPG alter LTD induction (*top right*). CPPG slightly reduced the degree of LTP achieved following TBS, but this failed to reach significance (*bottom right).*

3.2.2 Dopamine Receptors

In light of the substantial dopaminergic input that the perirhinal cortex receives from the VTA (Descarries *et al.*, 1987), the involvement of D_1 and D_2 dopamine receptors in perirhinal synaptic plasticity was assessed using both antagonists and agonists specific to either D_1-like or D_2-like dopamine receptors.

3.2.2.1 D_1-like receptors

The D_1 dopamine receptor antagonist SCH23390 hydrochloride (10 μM) (Schulz *et al.*, 1985) reduced the maximum EPSP amplitude recorded under basal stimulation (2.0±0.2 mV, n=14(3); P<0.01 two-tailed t-test) but did not alter the overall input-output profile (P>0.5 two-way ANOVA; figure 3.2.5). SCH23390 had a modest effect on paired-pulse depression (figure 3.2.11; figure 3.2.5) but failed to achieve statistical significance using two-way ANOVA (P>0.5).

SCH23390 did affect the induction of LTD, reducing the level of depression attained during conditioning and preventing the expression of LTD (6.0 ± 3.2%; n=6(2); significantly different than controls: −12.3±3.0%, n=20(9); P<0.01; two-tailed-unpaired t-test; figure 3.2.5). In contrast, the D_1 antagonist did not, affect LTP (18.8±4.8%; n=7(2); P>0.5, unpaired, two-tailed Welch-test; Figure 3.2.5).

The affect of a D_1 agonist (SKF38393, 10 μM) (Sibley *et al.*, 1982) on LTP induction was investigated and found to block induction in 5 out of 6 experiments (2.6±3.2%; n=6(3)). This degree of potentiation is significantly different than controls (18.4 ± 4.7%; n=11(6); P<0.02, unpaired, two-tailed Welch-test; summarised in figure 3.2.11).

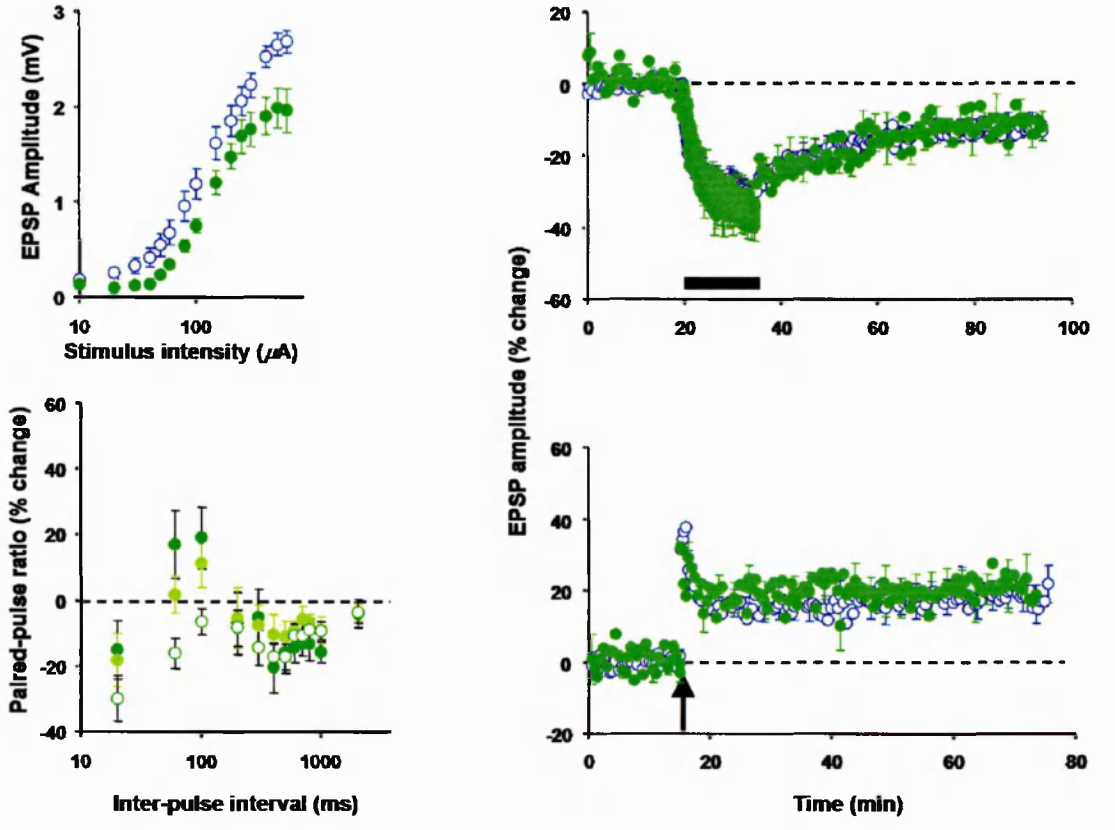


Figure 3.2.5 D_1 dopamine receptor dependency of synaptic plasticity in the mouse perirhinal cortex. SCH23390 reduced the input-output relationship (*top left*) but had little effect on paired-pulse outcome (b*ottom left)*, long-term depression (*tope right)* or long-term potentiation (*bottom right*).

3.2.2.2 D_2-like receptors

The typical D_2 dopamine receptor antagonist sulpiride (10 μM) (Seeman & Van Tol, 1994) was initially used in examining the involvement of D_2 dopamine receptors in perirhinal cortical plasticity. While this drug appeared to alter the paired-pulse profile towards that of facilitation, the precise action of sulpiride was masked by its solvent, ethanol. When ethanol alone was present in ACSF there was a similar shift in profile towards PPF. The water-soluble D_2 dopamine receptor-specific antagonist Remoxipride hydrochloride (10 μM) (Mohell *et al.*, 1993) was therefore used and was found to alter synaptic plasticity.

Remoxipride significantly increased the maximum amplitude evoked by test-shocks (3.2±0.2 mV, n=12(3); $P<0.05$, two-tailed t-test) but did not alter the overall profile of the input-output relationship ($P>0.2$ two-way ANOVA; figure 3.2.6). Remoxipride had a more overt affect on the paired-pulse profile than the D_1 dopamine receptor antagonist. The paired-pulse profile was again shifted towards facilitation (figure 3.2.6) and was significant at the 30% and 80% stimulus intensities ($P<0.01$; $F_{11,657}=2.68$ and $P<0.001$; $F_{11,645}=2.98$ respectively; two-way ANOVA). Remoxipride also blocked the induction of LTD in 6/8 slices (–0.3 ± 4.3%, n=8(3); significantly different than controls: –12.3±3.0%, n=20(9); $P<0.03$, unpaired two-tailed t-test; figure 3.2.6). Interestingly, the degree of frequency-dependent depression seen during the conditioning period was enhanced compared with control slices. When TBS was applied to superficial afferents in the presence of Remoxipride, LTP was still reliably induced (22.3±6.1%, n=9(3); $P<0.01$; two-tailed paired t-test) and was not significantly different than control ($P>0.5$ two-tailed t-test).

The D_2 dopamine receptor-specific agonist Quinpirole (10 μM) was applied to slices and the induction of LTD and LTP examined. While Quinpirole had little effect on LTD induction (4/7 slices; –11.7±6.8%; P>0.5, unpaired two-tailed t-test, n=7(3); figure 3.2.7) the agonist significantly increased the degree of potentiation achieved by TBS which induced LTP in 100% of experiments (39.1±6.8%; significantly different than controls: 18.4 ± 4.7%; n=11(6); P<0.05 unpaired two-tailed t-test, n=7(3); figure 3.2.7).

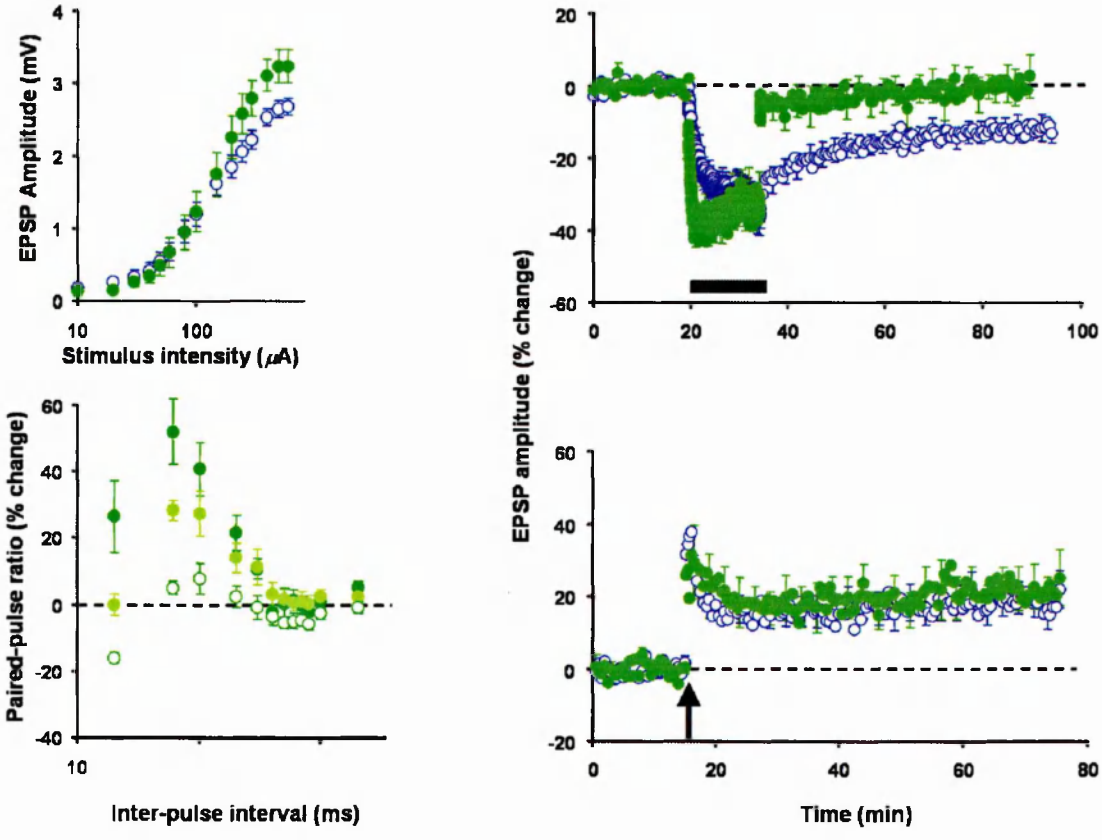


Figure 3.2.6 Antagonising D_2 dopamine receptors blocks synaptic depression. *Top left* The D_2 dopamine receptor antagonist Remoxipride increased the maximum amplitude of field responses. *Bottom Left* Remoxipride blocked PPD, resulting in the expression of PPF. *Top right* LTD induction was prevented by the antagonist, while LTP was unaffected (*bottom right*).

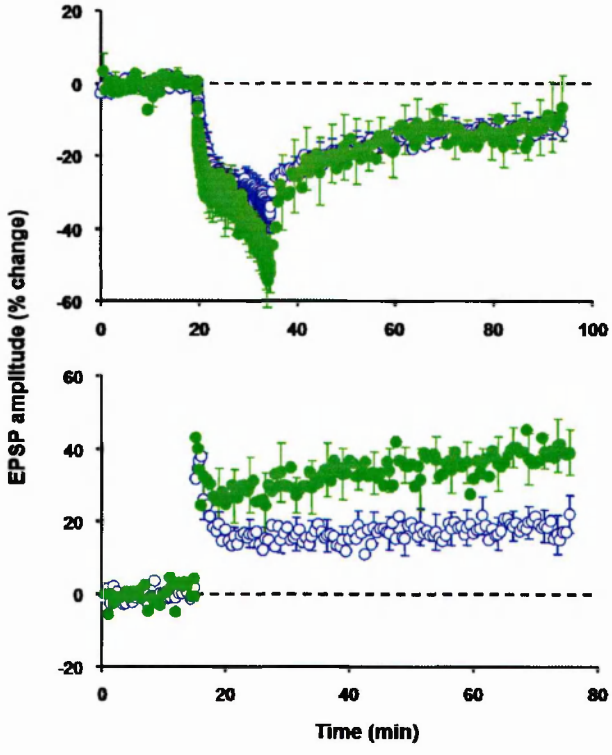


Figure 3.2.7 Perirhinal cortical long-term synaptic plasticity in the D_2 dopamine receptor antagonist Quinpirole. 10 μM Quinpirole has no effect on LTD induction (*top*), while enhancing LTP (*bottom*).

3.2.2.3 Co-application of dopamine antagonists

In an attempt to resolve the apparent disparity between the actions of D_1 and D_2 dopamine receptor antagonists on synaptic plasticity within the perirhinal cortex, both agents were co-applied to slices. Curiously, the co-application of the two dopamine receptor antagonists resulted in the full recovery of PPD at superficial synapses such that profiles were indistinguishable from controls ($P>0.5$; two-way ANOVA; figure 3.2.8). Furthermore, there was a partial recovery in the ability to express LTD (–9.1 ± 4.9%; n=9(3); figure 3.2.8), though this failed to achieve statistical significance.

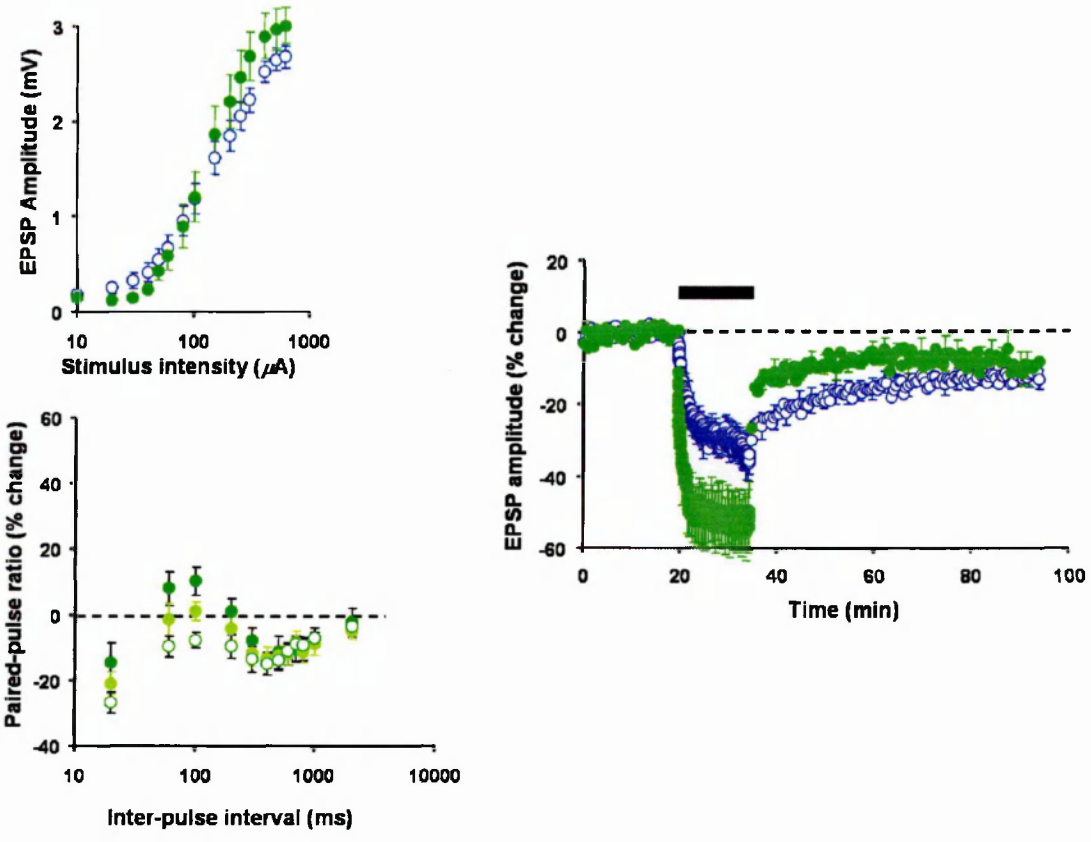


Figure 3.2.8 Co-application of both D_1 and D_2 dopamine receptor antagonists restores normal synaptic plasticity. *Top left* Input-output relationships are normal. *Bottom left* Paired-pulse profiles show normal PPD ratios. *Right* While a significant LTD is not induced, in the presence of both drugs, LFS showed a trend towards depression.

3.2.2.4 Dopamine receptors are located postsynaptically

Sharp intracellular recordings were used to identify cellular events following activation of dopamine receptors on pyramidal cells in layer II/III of the perirhinal cortex. This technique also demonstrates the postsynaptic localisation of these receptors. An example experiment is shown in figure 3.2.9.

Following impalement of a cell, input resistance of the individual cell was obtained and a stable E_m baseline recorded (–75.7±2.0 mV; n=3 cells). Bath application of the D_2 receptor agonist Quinpirole (10 μM) led to hyperpolarisation of the cell membrane potential (–8.8±2.1 mV; $P<0.03$, paired-t-test; n=3 cells). Hyperpolarisation was associated with a reduced input resistance from 49.7±3.3 MΩ to 40.8±3.1 MΩ ($P<0.001$, paired t-test; n=3 cells). Washing off the drug led to re-polarisation of the cell back to –78±1.9 mV and a return of the input resistance to 47.5±3.4 MΩ. Replotting the I/V data as absolute membrane potential against current injection created an intersect at approximately –120 mV, indicating a change in potassium conductance.

Conversely, application of the D_1 dopamine receptor agonist SKF38393 (10 μM) led to a slight, but insignificant, depolarisation (+1.4±2.4 mV) that was associated with a small (but again insignificant) increase in input resistance (+3.5±2.5 MΩ).

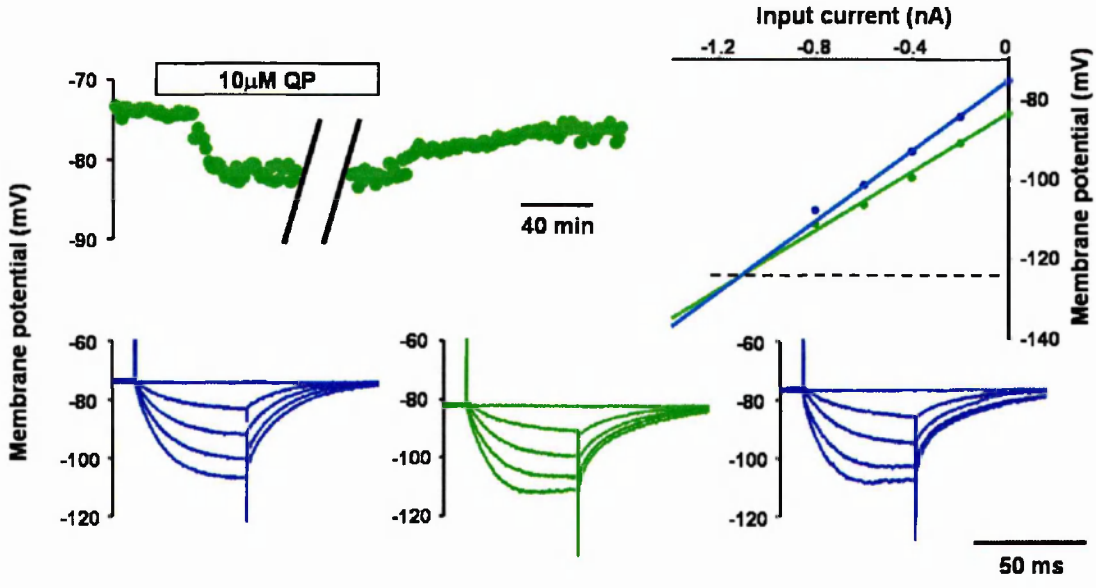


Figure 3.2.9 D_2 dopamine receptors are expressed postsynaptically. *Top left* The D_2 dopamine receptor agonist Quinpirole reversibly hyperpolarises layer II/III perirhinal neurones. *Bottom* Membrane responses to hyperpolarising current injections before (*left*), in the presence of Quinpirole (*middle*) and after washout (*right*). Input resistance was reduced in the presence of the drug and returned to baseline values following washout. *Top right* The cross over of the best fit lines (R^2>0.98) indicates an increased potassium conductance in response to D_2 receptor activation.

3.2.3 GABA receptors

Antagonists of GABA receptors were bath applied to slices in order to identify the importance of inhibitory activity in perirhinal cortical plasticity. Both $GABA_A$ and $GABA_B$ receptor antagonists alter the paired-pulse profile and impair LTD.

3.2.3.1 $GABA_A$

Antagonising $GABA_A$ receptors using bicuculline methiodide (BMI; 10 μM) increased the input-output relationship such that larger EPSP amplitudes were recorded at all stimulus intensities ($P<0.00001$, $F_{14, 1020}=3.881$, two-way ANOVA; figure 3.2.10). BMI enhanced paired-pulse depression at all intervals and produced profiles that were indistinguishable at 30, 50 and 80% stimulus intensities (figure 3.2.10). The presence of BMI did not, however, facilitate the induction of LTD. During conditioning there was an initial depression of the response similar to that seen at the 1000 ms paired-pulse interval and responses remained at this level throughout conditioning. After conditioning, however, there was an immediate return to baseline levels followed by a slight potentiation during the first 30 minutes that reached a plateaux at 7.1±2.4% (60 minutes, n=10(5), figure 3.2.10). The degree of potentiation was not, however, significantly different than the non-conditioned pathway (1.7±2.7%; $P>0.1$, paired-t-test), while it was significantly different than control slices (–12.3±3.0%, n=20(9); $P=0.0001$, two-tailed unpaired t-test).

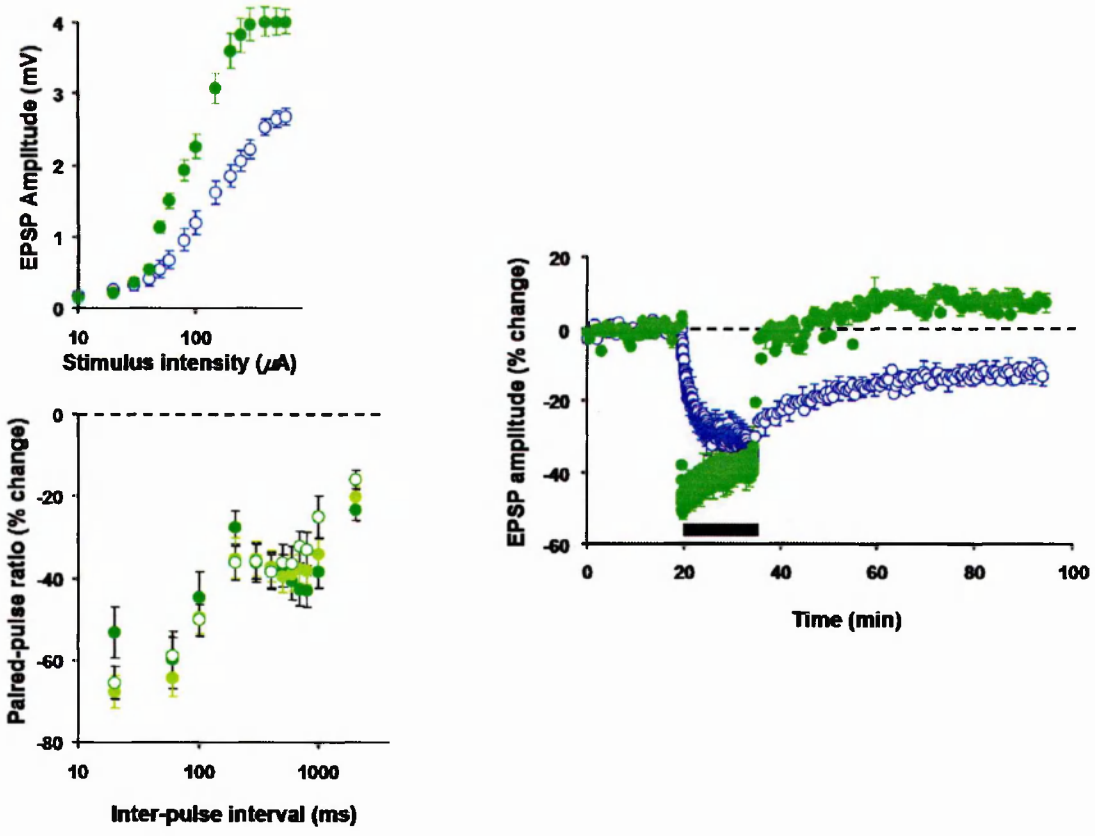


Figure 3.2.10 $GABA_A$ receptor dependency of synaptic plasticity in the mouse perirhinal cortex. *Top left* BMI increased the amplitude of the field potential. *Bottom left* Antagonising $GABA_A$ receptors enhanced the degree of PPD at each interval studied such that the profile obtained at each stimulus intensity was indistinguishable. *Right* Low-frequency stimulation led to a frequency-dependent depression, but responses returned to baseline following LFS and a slow-onset potentiation developed.

3.2.3.1 $GABA_B$

Antagonising $GABA_B$ receptors increased the maximum EPSP amplitude (3.5±0.2 mV; n=19(3); P<0.01, unpaired t-test; figure 3.2.11) and the overall input-output relationship was altered (P<0.0001, $F_{14, 960}$=3.2). Paired-pulse profiles were also altered (figure 3.2.11). At intervals of less than 100 ms there were no overt differences between control and drug-treated profiles, while paired-pulse depression was no longer expressed at intervals over 100 ms at all three stimulus intensities (P<0.001 at all three intensities, two-way ANOVA). In the presence of SCH50911, LFS led to impaired frequency dependent depression during conditioning. Immediately after conditioning, responses returned to baseline amplitude followed by a slow-onset depression which returned to baseline within one hour (–9.7±3.1% at 30 minutes; +1.1±3.0% at 60 minutes).

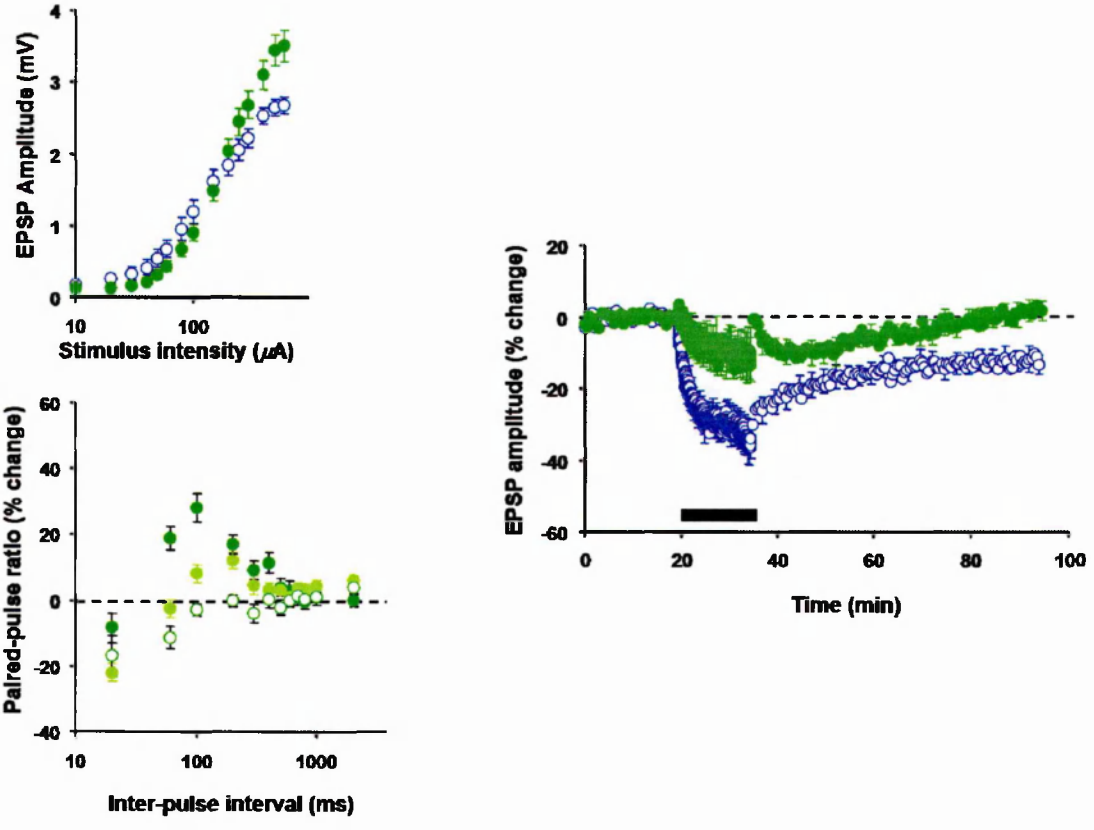


Figure 3.2.11 $GABA_B$ receptor dependency of synaptic plasticity in the mouse perirhinal cortex. *Top left* An antagonist of $GABA_B$ receptors increased EPSP amplitude at the larger stimulation intensities. *Bottom left* $GABA_B$ receptors are important for the expression of PPD at intervals greater than 200 ms. *Right* Blockade of $GABA_B$ receptors reduces the degree of frequency-dependent depression achieved during LFS. Immediately following conditioning, responses returned to baseline and a slow-onset, short-lasting depression was seen.

3.2.4 Summary

The neurotransmitter receptor-dependency of mouse perirhinal cortical plasticity is summarised in figure 3.2.12. The receptor-dependency of synaptic plasticity in the mouse perirhinal cortex is interesting. While showing many similarities to the rat perirhinal cortex, there are some key differences. Namely, unlike in the rat, LTD in the mouse does not appear to be NMDA receptor-dependent; in contrast, like the rat, LTD induction *is* dependent on group I and II mGlu receptor activation, but not group III. Interestingly a similar receptor dependency is seen for short-term plasticity, which suggests that LTD in this brain region may, in part, have a presynaptic locus of expression.

The finding that synaptic plasticity within the perirhinal cortex is dependent on dopamine receptor activation is a novel finding and has important implications in the interpretation of synaptic transmission within this brain region. In particular, blockade of D_2 dopamine receptors blocks LTD induction and produces a pronounced change in paired-pulse profile, suggesting that activation of this class of dopamine receptor and the subsequent down regulation of cAMP is crucial for the expression of plasticity within the perirhinal cortex.

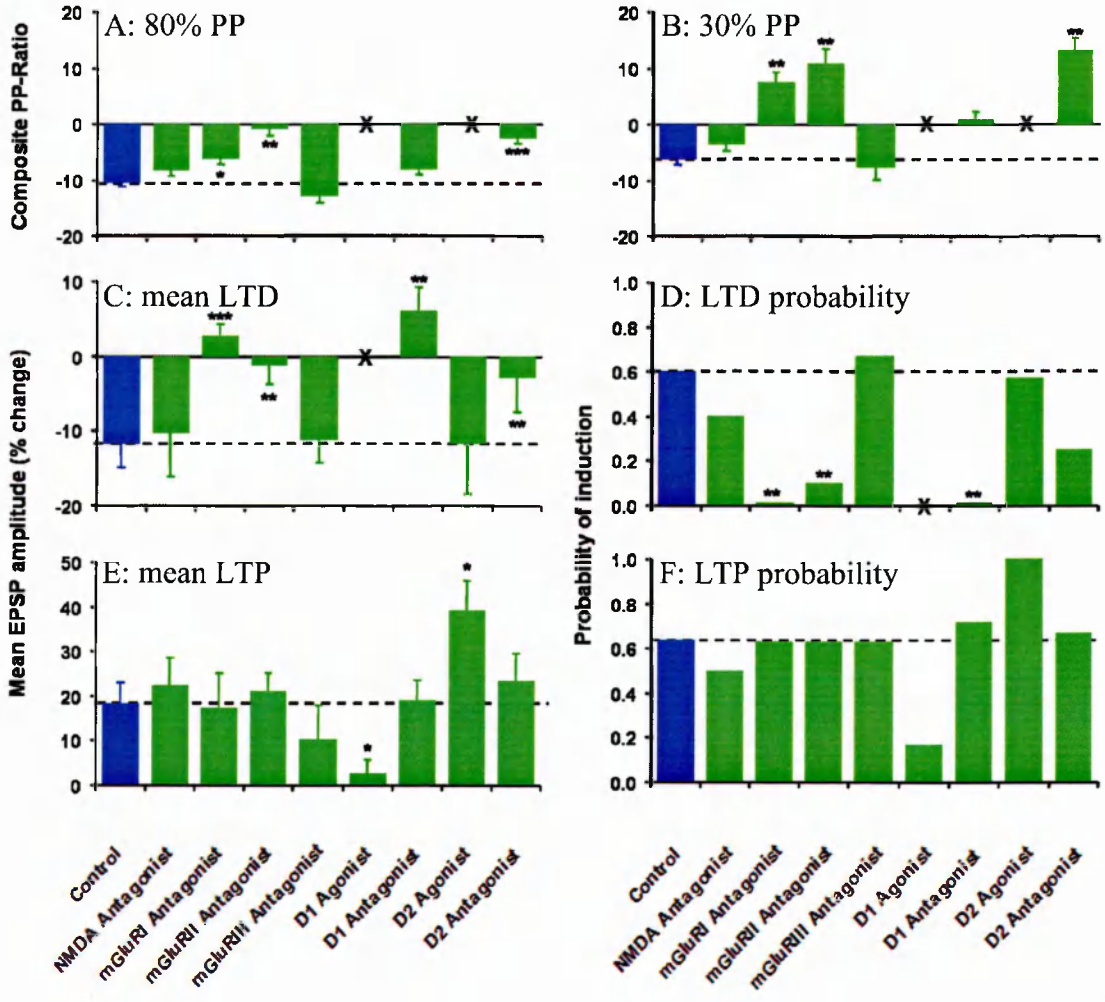


Figure 3.2.12 Summary of receptor dependency for perirhinal synaptic plasticity. *Top:* Composite paired-pulse ratios (see methods section 2.1.10) at 80 (*A*) and 30 (*B*) *percent* stimulation intensities. The mean ± SEM of the paired-pulse ratio was calculated from all inter-pulse intervals (20-2000 ms) recorded from each pathway, giving a value that represents the total degree of depression or facilitation in each condition. Statistics represent significance obtained using two-way ANOVA (see main text). *Middle:* Receptor-dependency of LTD induction. Mean EPSP amplitudes at 60 minutes post conditioning (C) and probabilities of inducing LTD in each condition (D). *Bottom* Receptor dependency of LTP induction. Mean EPSP amplitudes at 60 minutes post-conditioning (*E*) and probability of LTP induction in each condition (*F*). Dotted line in each chart represents control levels. Asterisks on the probability plots indicate significant differences using Fisher's Exact Test. **X** = no result.

3.3 Abnormal Synaptic plasticity in R6/1 mice

Following the characterisation of synaptic plasticity within the perirhinal cortex of CBA × C57BL/6 mice, a linear study of the R6/1 mouse was made. For these experiments, data obtained from transgenic slices were compared to age-matched wild type littermate controls.

3.3.1 Characterisation of the R6/1 phenotype

Aspects of the R6/1 phenotype were assessed as follows. Clasping was used as the key determinant of the R6/1 phenotype.

3.3.1.1 Weight

Mice were grouped by sex and genotype and their weights binned by age to the nearest month. Mean weight ± SEM was then plotted against age (figure 3.3.1). Transgenic males were significantly underweight compared to age- and sex-matched littermate controls at five months of age and older. In contrast, female transgenic mice did not show a significant loss of weight, although they did show a trend to be lighter than wild types. Upon full dissection of male transgenic mice, it was noted that they appeared to have comparable fat deposits, suggesting that the loss in body weight may be at the expense of muscle tissue.

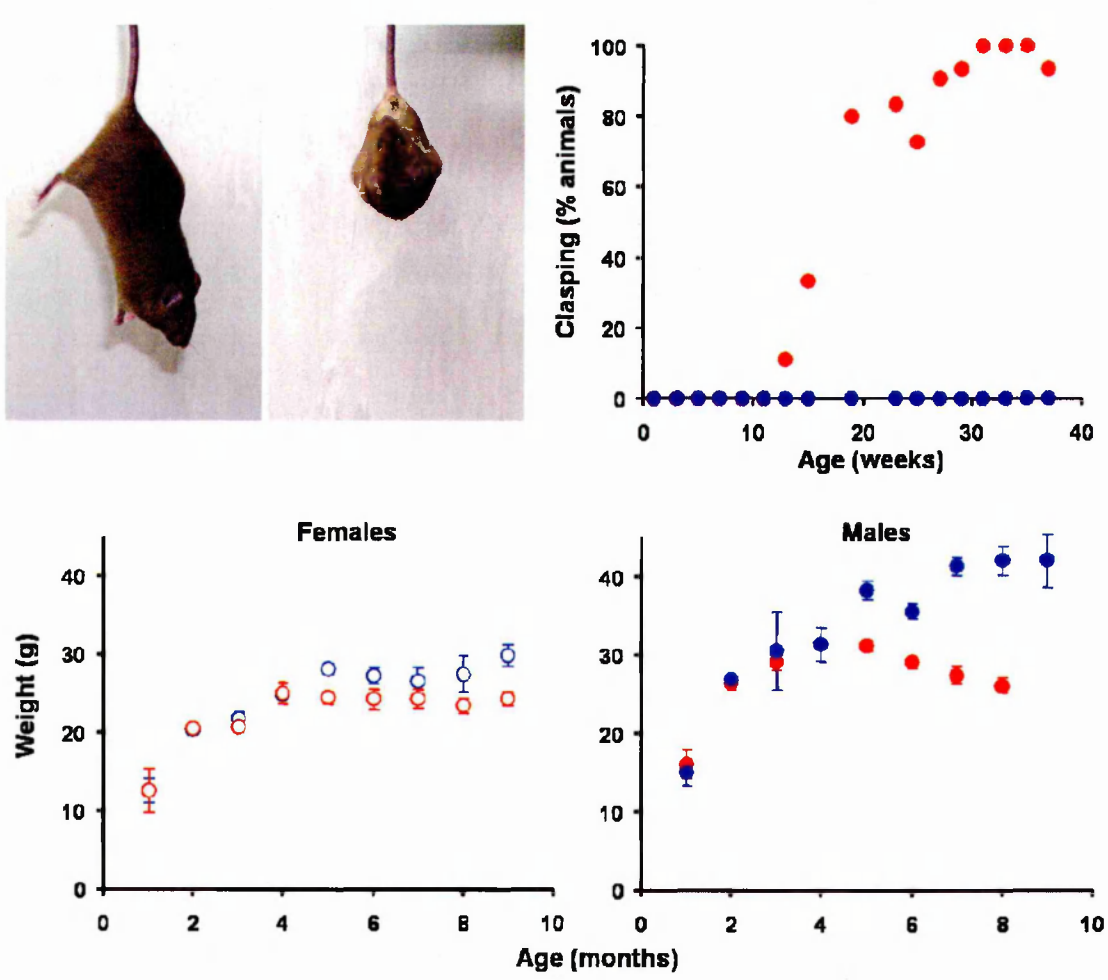


Figure 3.3.1 Characterisation of the R6/1 gross phenotype. *Top left* Photographs showing the clasping phenotype in an eleven month old R6/1 mouse (right) and its absence in an age-matched littermate (left). *Top right* Percent of animals showing the clasping trait at each age (● wild type; ● transgenic) *Bottom left* Comparison of body mass of female transgenic and wild type mice. *Bottom right* Body mass of male animals. Males show a divergence from approximately 5 months of age ($P<0.001$, two-tailed t-test).

3.3.1.2 Clasping

The clasping phenotype was assessed in mice from 1 week up to 37 weeks of age. Clasping was never seen in wild-type mice. Clasping was observed in transgenic mice from the age of 13 weeks and by 19 weeks over 80% of animals were clasping in at least 3/10 trials. Figure 3.3.1 shows the clasping phenotype and summarises the onset of this motor defect.

3.3.2 Electrophysiological phenotype

The cellular and synaptic phenotypes of R6/1 perirhinal cortex were investigated in asymptomatic mice (2 month), mice that were just starting to show neurological symptoms (5 months), symptomatic (7 months) and nearing end-stage (9 months) (Home Office regulations prevented the study of end-stage mice).

3.3.2.1 Cellular properties

It was noted that it was generally easier to find and impale layer II/III perirhinal cells in transgenic slices than in wild type slices. At 3 months of age, cells in transgenic slices showed a normal resting membrane potential (E_m= –79.4±1.0 mV, n=10). At 5 months, however, there were two discernable populations of cells. The first showed a normal resting membrane potential (–77.5±0.9 mV, n=6), the second

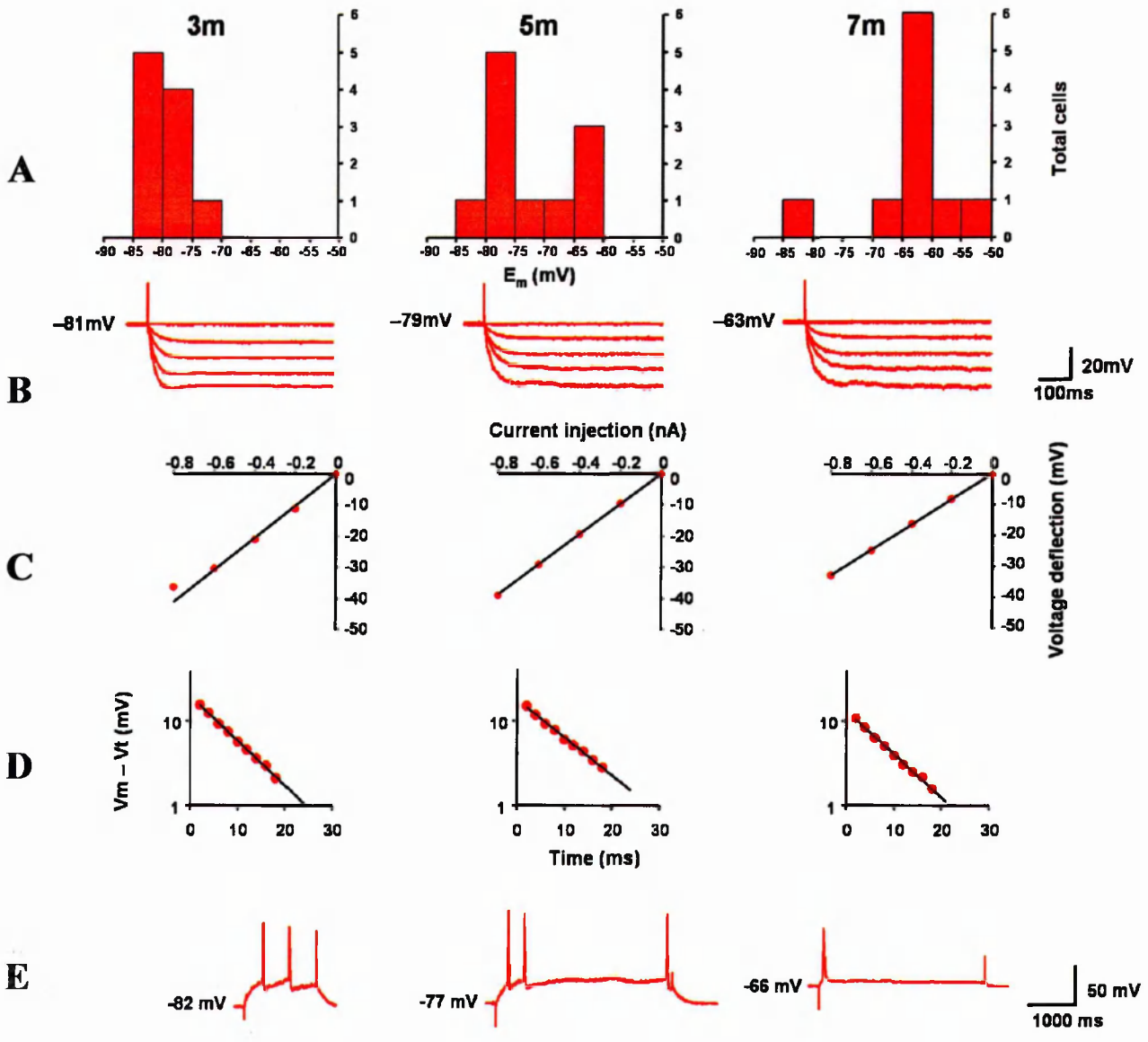


Figure 3.3.2 Progressive alterations in the passive membrane properties of R6/1 perirhinal cortical neurones. *Row A* Distributions of the resting membrane potentials of layer II/III perirhinal neurones at 3, 5 and 7 months of age. At 3 months old, neurones show a similar resting membrane potential to neurones in the CBA×C57BL/6 mice. At 5 months, however, two subpopulations were identified. Some cells showed a normal resting membrane potential, the other had a depolarised resting membrane potential. By 7 months the majority of cells had a depolarised resting membrane potential. *Rows B & C* Current-voltage relationships in response to negative current injections. At both 3 and 5 months old input resistances were higher than in control experiments, but were within the normal range. At 7 months, R_{in} was increased above that of either 3 or 5 months old cells. *Row D* Time courses of the hyperpolarising membrane response to a −0.4 nA current injection. At 3 and 5 months old, τ_m was similar to control mice, while at 7 months old, the membrane time constant was reduced. *Row E* Cellular responses to positive current injection. At 3 months action potential generation appeared normal, while at 5 and 7 months, action potentials fired irregularly and had smaller amplitude.

Cellular property	Mean ± SEM		
	3 month n=6	**5 month n=7**	**7 month n=5**
E_m	−79.4 ± 1.0 mV [a]	−69.5 ± 2.5 mV [b]	−63.1 ± 2.6 mV [c]
R_{in}	42.5 ± 2.5 MΩ	47.5 ± 2.5 MΩ	57.8 ± 6.7 MΩ***
τ_m	10.0 ± 0.3 ms	9.8 ± 0.5 ms	6.6 ± 0.4 ms
C_m	0.23 ± 0.02 nF	0.21 ± 0.02 nF	0.12 ± 0.01 nF*
$AP_{threshold}$	−47.3 ± 2.5 mV	−51.4 ± 4.5 mV	−52.9 ± 3.9 mV
$AP_{overshoot}$	+28.2 ± 1.5 mV	+27.3 ± 5.4 mV	+5.2 ± 2.1 mV
$AP_{amplitude}$	75.7 ± 1.6 mV	78.7 ± 6.9 mV	60.0 ± 2.1 mV*
AP_{width}	1.73 ± 0.10 ms	2.75 ± 0.48 ms	4.05 ± 0.21 ms*

Table 3.3.1 Cellular properties of layer II/III perirhinal neurones in R6/1 transgenic mice. Asterisks denote significant difference against previous age group. [a] n = 10 cells. [b] over all mean, in reality there are two sub-populations, one showing normal E_m the other with depolarised E_m (see main text); n = 11 cells. [c] n = 10 cells. n = Number of cells.

showed a depolarised membrane state (–64.8±2.5 mV, n=5). By 7 months of age 90% of cells showed a depolarised resting membrane potential (–63.1±2.6, n=10).

Both wild type and transgenic cells showed membrane hyperpolarisation in response to a negative current injection (–0.2 to –0.8 nA). There was an initial charging of the membrane until a steady state was reached. Current-voltage plots highlighted a progressive increase in input resistance in the transgenic slices. At 3 months of age, R_{in} values were not significantly different than those obtained in the control study (42.5±2.5 MΩ; P>0.5; n=6). At 5 months there was a trend towards an increased input resistance (47.5±2.5 MΩ, n=7) but this failed to reach significance (P=0.14). At 7 months of age there was a significant increase in input resistance (57.8±6.7 MΩ, P<0.001, two-tailed t-test; n=5).

The membrane time constant (τ_m) was found to be normal at both 3 (10.0±0.3 ms; P>0.5) and 5 (9.8±0.5 ms; P>0.5) months of age. At 7 months, there was a trend for τ_m to be reduced (6.6±0.4 ms) but this did not reach significance (P=0.19). Membrane capacitance was calculated from R_{in} and τ_m. At both 3 and 5 months, C_m was similar to controls (0.23±0.02 nF; 0.21±0.02 nF respectively; P>0.5 in both conditions). At seven months of age membrane capacitance was significantly reduced by as much as 50% compared to the control experiments (0.12±0.01 nF; P<0.05).

Injection of positive current (+0.2 to +0.8 nA) depolarised the cell and evoked action potentials in both wild type and transgenic slices. Action potentials were analysed at 3, 5 and 7 months of age and action potential threshold, overshoot, amplitude and duration (at half-maximum amplitude) were ascertained (table 3.3.1). Threshold was not significantly altered at any age, while overshoot was reduced at 7

months, leading to a significant reduction in action potential amplitude (60.0±2.1 mV; $P<0.05$; n=7 cells). The duration of the action potential was also found to be significantly increased at 7 months ($P<0.05$ vs. 5 month-old and $P<0.00001$ vs. 1 month-old; two-tailed t-test).

3.3.2.2 Field responses

The field potential recorded from wild type slices appeared to be identical to that seen in the background strain. In contrast, the R6/1 perirhinal cortical field potential was markedly different (figure 3.3.3; figure 3.3.4). Strikingly, a prolonged late component (onset latency of ~9.4 ms) is seen in the majority of transgenic slices that is absent in wild type potentials. This late component was seen in slices prepared from transgenic animals of all ages. The late component was not susceptible to pharmacological manipulation and persisted in the presence of the NMDA receptor antagonist D-AP5 (50 μM; figure 3.3.4); the group II mGlu receptor agonist DCG-IV (10 μM); the D_1 dopamine receptor antagonist SCH23390 (10μM) or agonist SKF38393 (10 μM); the D_2 dopamine receptor antagonist Remoxipride (10μM) or agonist Quinpirole (10 μM; figure 3.3.4); and also the $GABA_A$ receptor antagonist PTX (2.5 μM; figure 3.3.4).

In order to account for the late component, EPSP area was used to measure the field potential in all experiments investigating LTD that are reported in section 3.3.

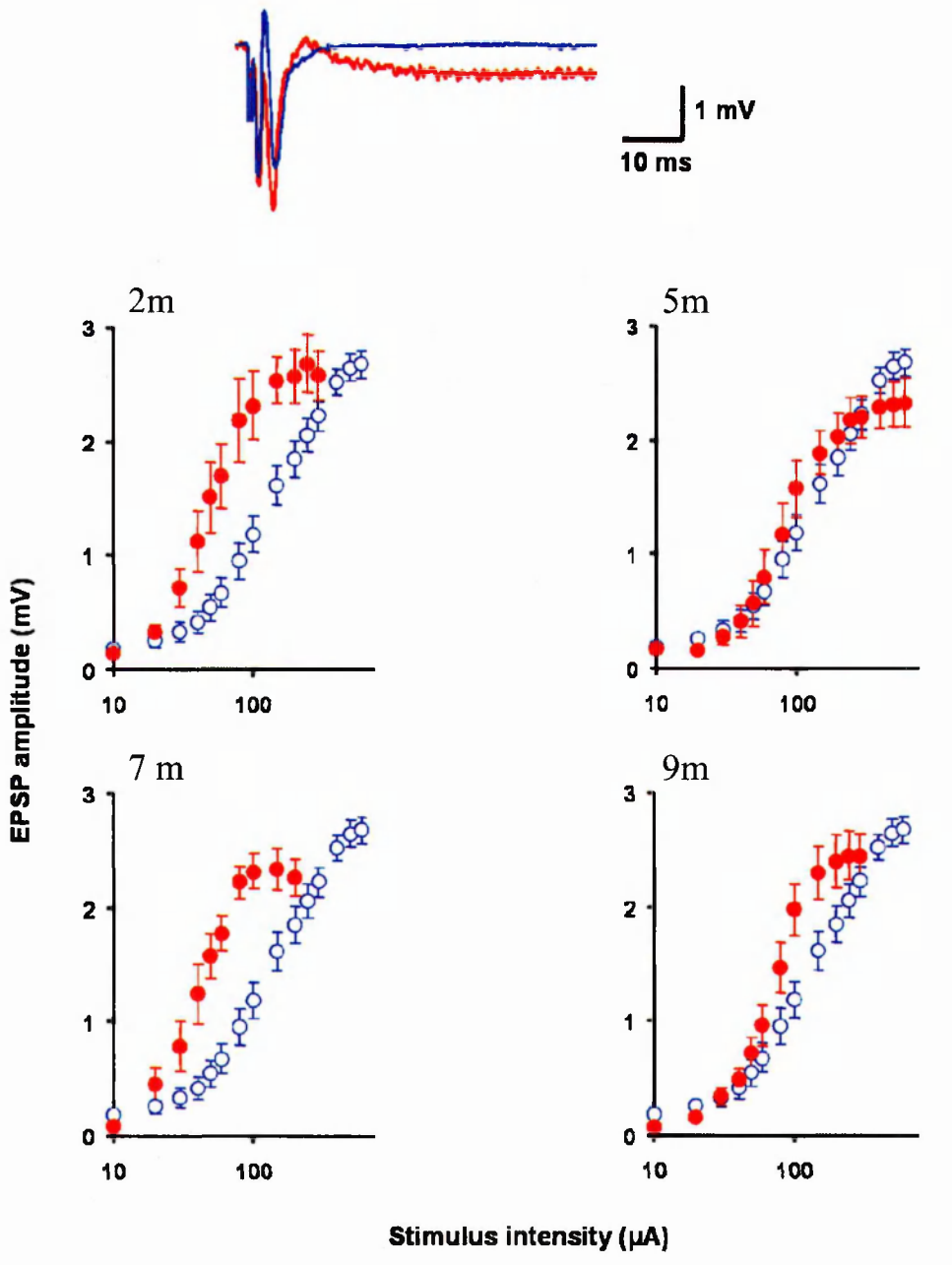


Figure 3.3.3 Progressive alterations in the input-output relationship in transgenic mice. The input-output relationship for transgenic mice aged 3, 5, 7 and 9 months old are represented by • (in all subsequent figures, results obtained from transgenic animals are shown in red). For comparison only, input-output relationships from the wild-type animals at 2 months of age are also shown (○). An example trace from a slice prepared from a 7 month old transgenic mouse is shown above compared to wild type (average of five traces).

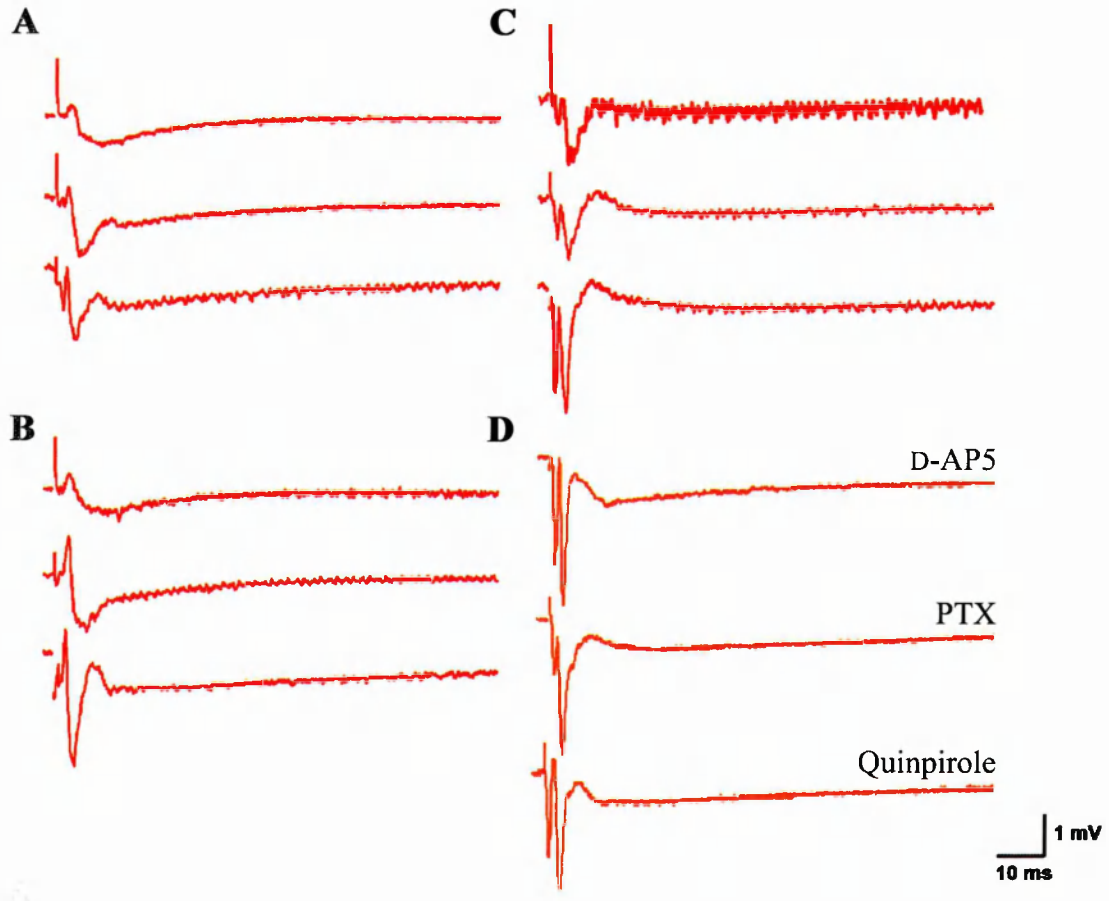


Figure 3.3.4 Example field potentials recorded from the R6/1 perirhinal cortex. *A-C* Representative EPSPs obtained at approximately 30, 50 and 80% maximal amplitude. Responses from slices prepared from two month old (**A**); five month old (**B**); and seven month old (**C**) animals. **D**: The NMDA receptor antagonist D-AP5 (top); $GABA_A$ receptor antagonist PTX (middle); or the D_2 dopamine receptor agonist Quinpirole (bottom) failed to affect the late component. N.B. traces in D are obtained from different slices. All traces are the average of 5 consecutive responses.

3.3.3 Plasticity

Both short-term and long-term plasticity was studied in the R6/1 perirhinal cortex and compared to age-matched littermate controls.

3.3.3.1 Paired-pulse

Paired-pulse stimuli were applied to sets of slices prepared from transgenic and wild type animals from each age group (figure 3.3.5; for clarity purposes, only the 80% paired-profiles have been shown; see figure 3.3.6 for profiles obtained at both 30 and 50% intensities). Wild-type slices exhibited paired-pulse depression profiles similar to those seen in the background strain, at all ages studied. At one to two months of age, slices showed a similar paired-pulse profile to wild types ($P>0.5$, two-way ANOVA). At 5 months there was a trend to show a lesser degree of depression, but this was not significant ($P>0.5$, two-way ANOVA). At seven months, however, paired-pulse facilitation was favoured over depression at all three stimulus intensities ($P<0.00001$, two-way ANOVA). At 9 months, facilitation was still favoured over depression, but PPF was not as large as that seen at 7 months. There was a significant difference between transgenic and wild type profiles at 80% stimulation intensity only ($P=0.008$, $F_{11,378} = 2.34$; two-way ANOVA).

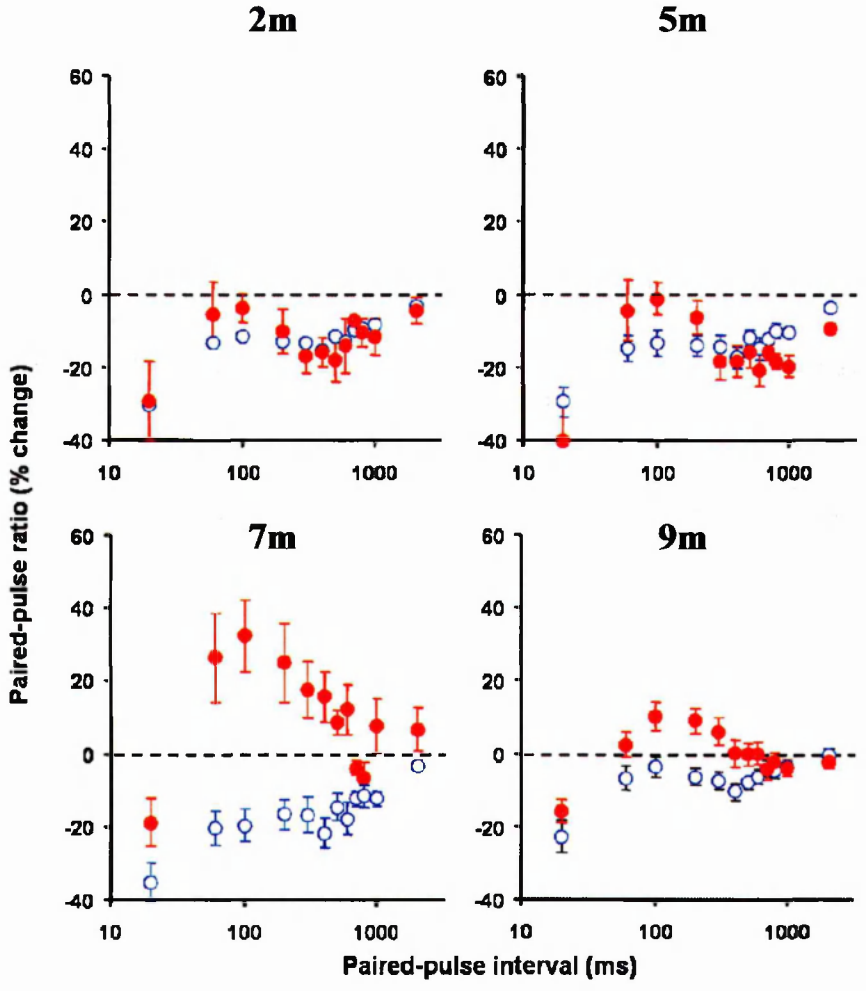


Figure 3.3.5 Progressive loss of paired-pulse depression in R6/1 perirhinal cortex. Paired-pulse profiles are shown for the 80% stimulation intensity. Profiles were obtained from 2, 5, 7 and 9 month old age groups. At both 2 and 5 moths of age, normal PPD is expressed. At 7 and 9 months of age, however, profiles show a significant shift towards that of facilitation. Aged-matched littermate controls are also shown. See also figure 3.3.6.

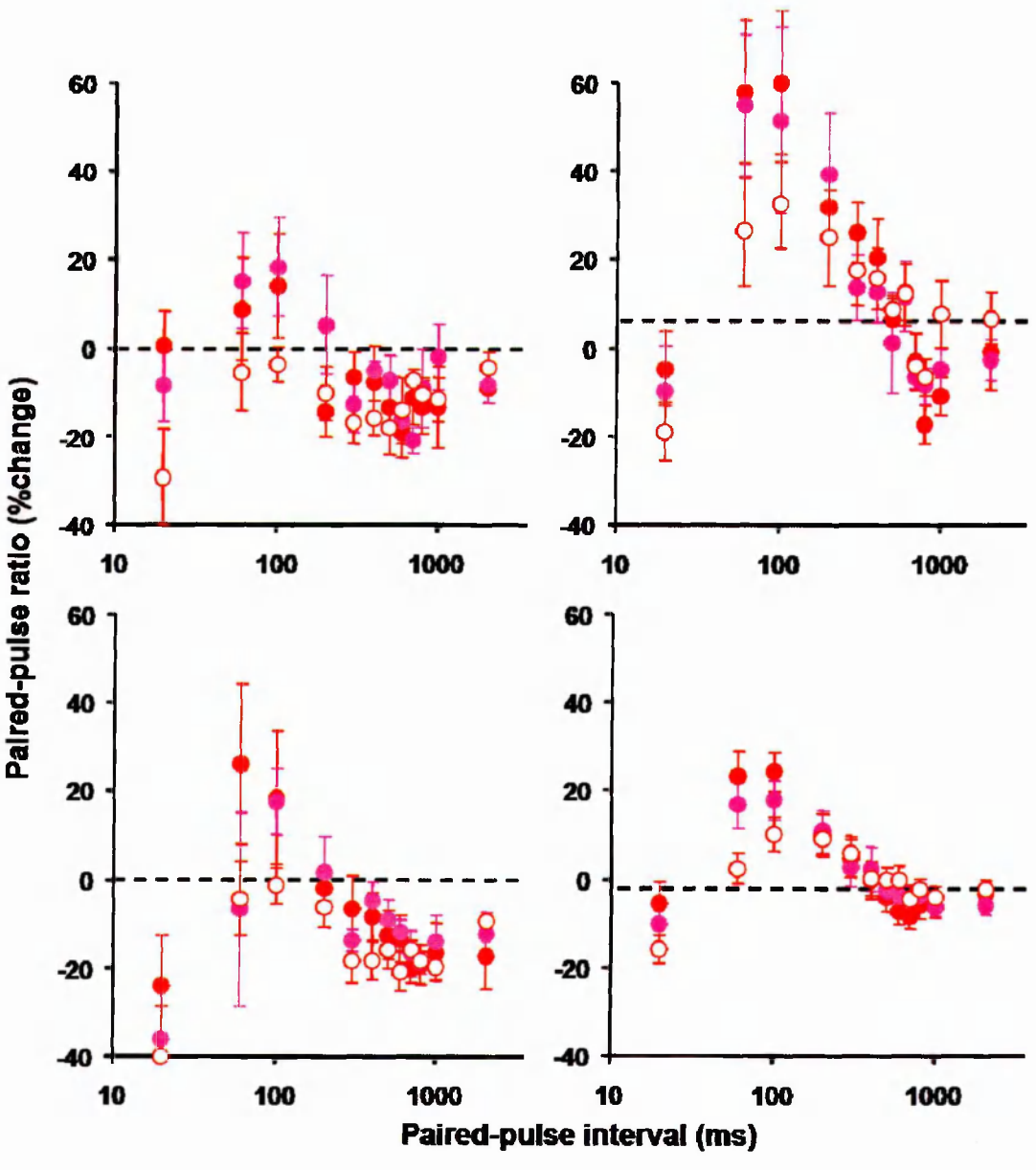


Figure 3.3.6 Outcomes of paired-pulse stimulation in the R6/1 perirhinal cortex. Profiles are shown at stimulation intensities set to evoke responses at 30 (●), 50 (●) and 80 (○) % maximal amplitude. Profiles were obtained in slices prepared from one month (*top left)*, five month (*bottom left*), seven month (*top right)* and nine month (*bottom right)* old mice.

3.3.3.2 LTD

Similarly to the paired-pulse analysis, there was a progressive loss of long-term depression at transgenic superficial perirhinal synapses, but not in wild types (shown in figure 3.3.7 and summarised in figure 3.3.8). LTD induction in slices prepared from two month old transgenic animals was enhanced compared to their littermate controls (−42.7±14.8%; n=5(2) vs. −19.4±4.7%; n=16(6); $P<0.05$, unpaired, two-tailed t-test). At five months of age, LTD showed a trend towards a decrease, but this failed to reach significance compared to littermates (−10.9±5.9%; n=7(3) vs. −24.6±7.2%; n=7(3); $P>0.2$). By seven months, however, LTD was hardly ever induced (14% of experiments) and was highly significantly different than littermates (+8.1±6.8%; n=7(3) vs. −16.5±5.7%; n=6(3); $P<0.0001$, two-tailed t-test). At 9 months, LTD was never observed and again LFS gave rise to a small degree of potentiation (+7.3±6.0%; n=11(4)) while LFS applied to control slices resulted in LTD (−30.0±9.9%, n=7(3)). The difference between wild type and transgenic LFS outcome was significant to $P<0.003$ (two-tailed t-test).

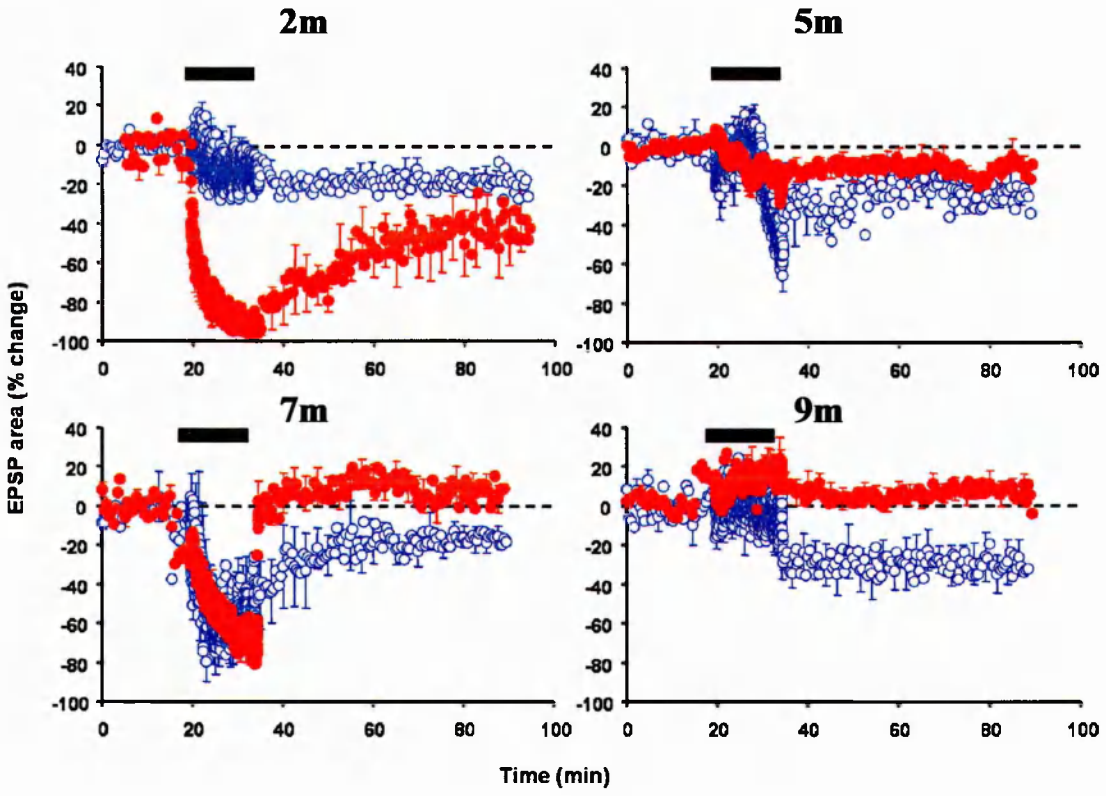


Figure 3.3.7 LTD expression in R6/1 perirhinal cortex. The progressive change in the outcome of LFS is clearly seen. At 2 moths of age LFS gives rise to an enhanced LTD with respect to littermate controls. At 5 months there is a slight, but insignificant reduction. At 7 and 9 months of age LTD is not expressed, while wild-type mice maintain LTD expression at all ages.

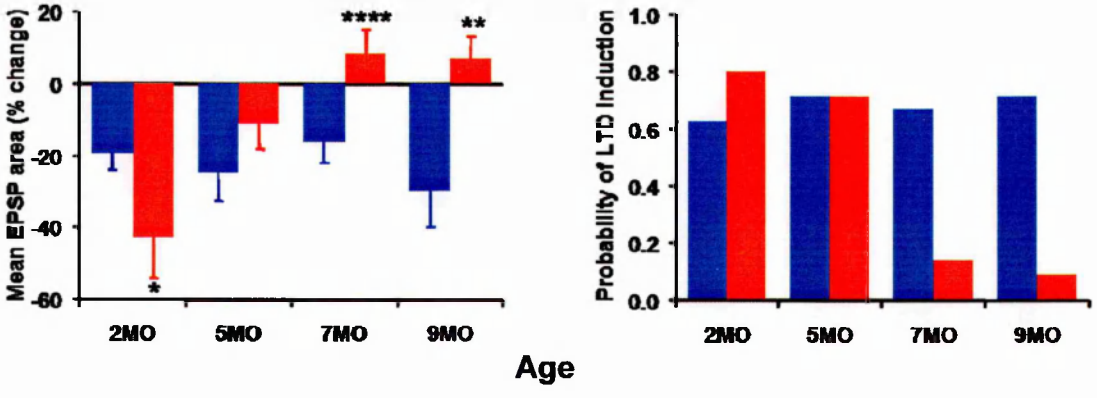


Figure 3.3.8 Summary of LTD induction in the R6/1 perirhinal cortex. *Left* Mean LTD induced in wild type (blue) and transgenic (red) slices at the ages indicated. Asterisks indicate significant differences between transgenic and age-matched littermates. *Right* Probability of LTD induction in transgenic and wild type slices.

3.3.3.3 Spike LTD

Spike depression was investigated in slices prepared from seven-month-old transgenic animals using intracellular recording techniques. During baseline recordings, test-shocks evoked an action potential in >90% of trials (example shown in figure 3.3.9; n=2(2)). During LFS, spikes were observed during the first ~1 minute of conditioning and then subthreshold responses were evoked. Following LFS there was a period of post-conditioning depression, but firing recovered within 5 minutes and continued to fire in >76% of trials. Conditioning, however, led to a potentiated component to the response immediately after the action potential (figure 3.3.9).

In an attempt to recapitulate the R6/1 conditions in a wild type slice, a wild-type cell was depolarised to approximately –66 mV during a conditioning train of synaptic inputs. This, however, did not prevent the induction of spike LTD as, following conditioning, the cell ceased to fire action potentials (figure 3.3.9).

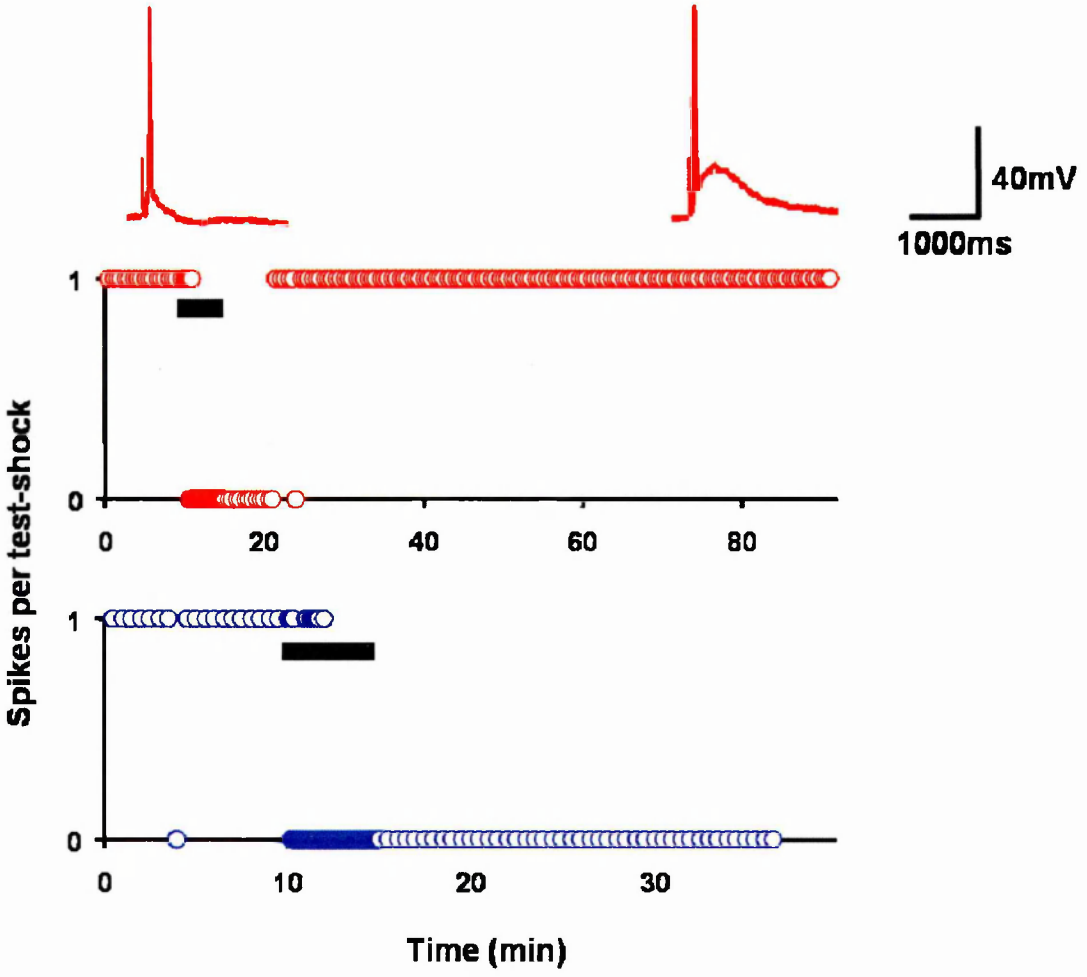


Figure 3.3.9 Seven month old transgenic slices do not show spike depression. *Top* Lack of spike-LTD in transgenic animals. During baseline recording the cell in this example fired reliably upon application of test-shocks. Following conditioning there was a period of depression and then a return to action potential firing. Example traces from baseline and 60 minutes after conditioning are shown above (single traces). *Bottom* Depolarising a wild-type cell to −66 mV during conditioning did not prevent the induction of spike-LTD. Note the different time scales.

3.4 Recovering normal synaptic plasticity

There is a marked loss of dopamine receptors within the brains of HD gene carriers prior to the onset of a neurological phenotype (Andrews *et al.*, 1999) and a similar reduction is seen in the R6/2 mouse brain (Cha *et al.*, 1998). Agonists of dopamine receptors were therefore applied to transgenic slices to investigate if a decrease in perirhinal expression of dopamine receptors could underlie the altered synaptic phenotype.

3.4.1 D_2 dopamine receptors

The D_2 dopamine receptor antagonist Quinpirole was bath applied to transgenic slices and synaptic plasticity investigated. Interestingly, Quinpirole reduced the degree of paired-pulse facilitation observed in 5 and 7 month old transgenic animals (figure 3.4.2; see also figure 3.4.1) (9 months not tested) such that PPD of drug-treated slices was not significantly different than that of wild type mice ($P>0.3$, two-way ANOVA). Quinpirole had no effect on paired-pulse profiles in wild-type slices.

Quinpirole also affected the expression of LTD (figure 3.4.2). LTD was enhanced at 5 months with respect to untreated transgenic slices (–31.3±4.1%; $P<0.02$ n=6(2)) and the degree of LTD induced in the presence of the drug was indistinguishable from wild type slices ($P>0.3$; unpaired t-test). Quinpirole also restored LTD expression at 7 months of age (–42.1±13.7%; $P<0.01$, paired t-test; n=9(3)). In the presence of the drug, LTD was significantly different than untreated slices ($P<0.02$) and not significantly different than age-matched littermate controls ($P>0.1$). Importantly, as discussed earlier, Quinpirole did not affect LTD induction in wild type slices.

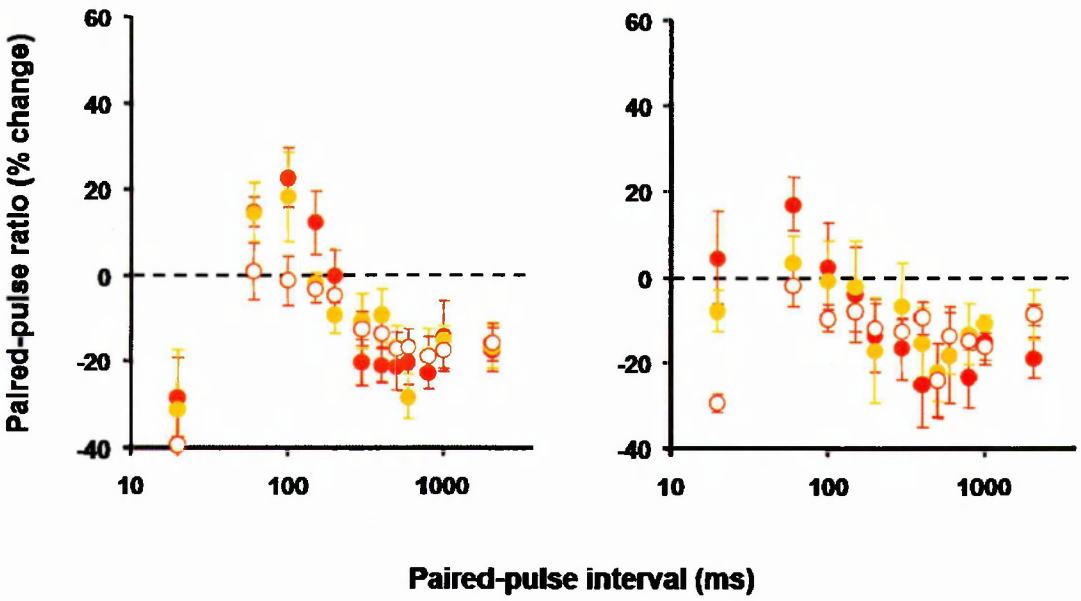


Figure 3.4.1 Quinpirole recovery of PPD in transgenic slices. The D_2 dopamine receptor agonist Quinpirole restores normal PPD in transgenic slices prepared from five (*left*) and seven (*right*) month old R6/1 mice. Profiles are shown at 30 (●), 50 (●) and 80 (○) *per cent* maximal stimulation.

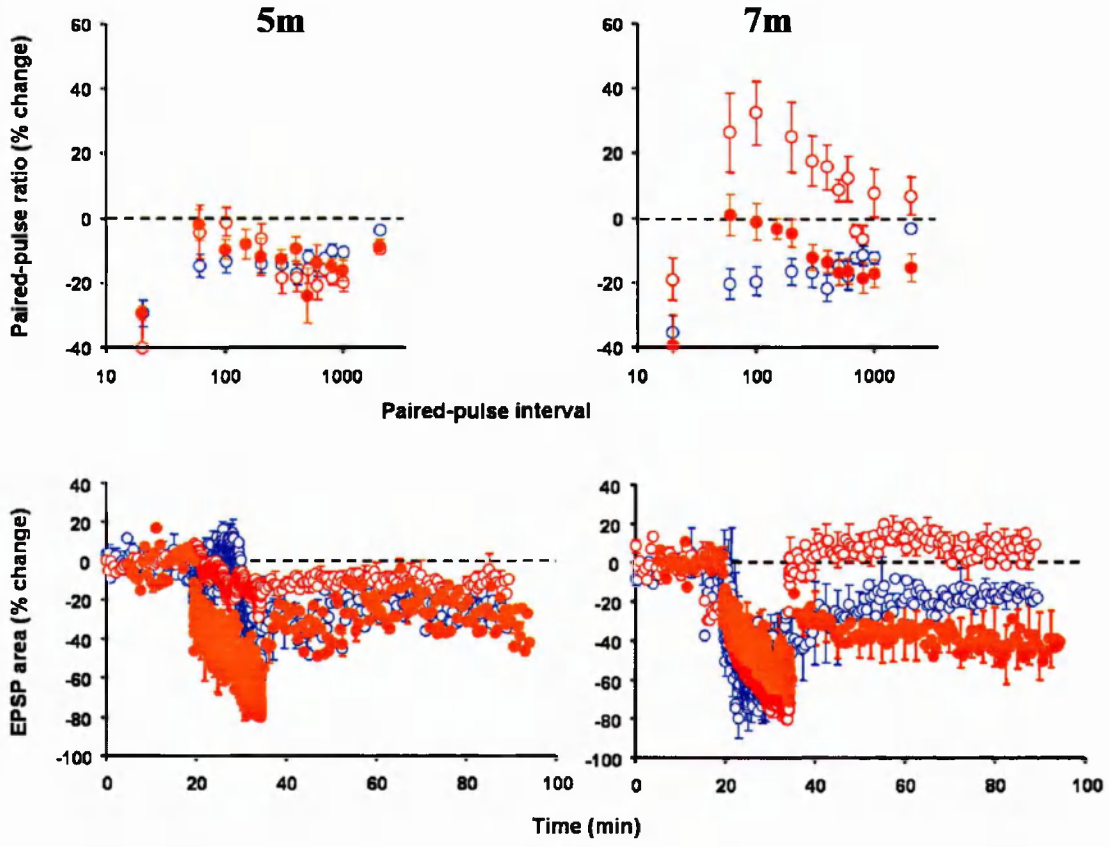


Figure 3.4.2 Quinpirole recovers normal synaptic plasticity at transgenic perirhinal synapses. The D_2 dopamine receptor agonist Quinpirole (•) was found to recover paired-pulse depression (*top*; see also figure 3.4.1*)* and long-term depression (*bottom)* at transgenic synapses at both 5 and 7 months of age.

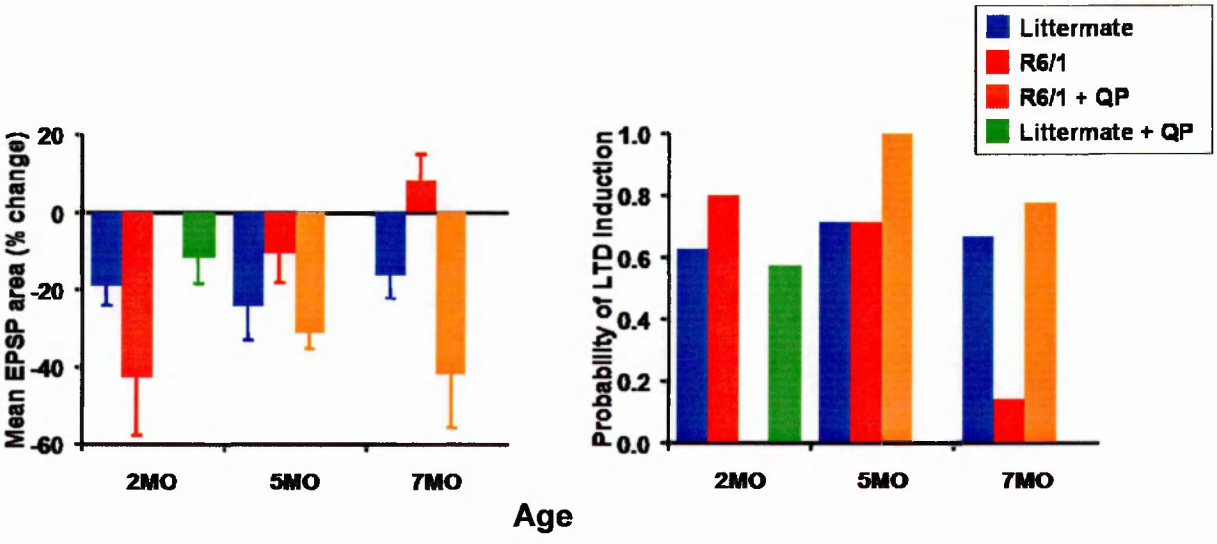


Figure 3.4.3 Summary chart showing Quinpirole recovery of LTD in transgenic animals. *Left* Mean EPSP amplitude 60 minutes after conditioning. *Right* Probability of LTD induction.

3.5 Immunohistochemistry

Immunohistochemical techniques were used to study the development of mutant huntingtin aggregation and dopamine receptor expression. Primary antibodies raised to the appropriate antigen were applied to slices prepared from animals aged 1, 3 and 7 months old. Aggregate formation was highlighted using a diaminobenzidine reaction and then slices viewed at the light level. A separate set of slices prepared from the same animals were exposed to antibodies raised to subclasses of D_1 and D_2 dopamine receptors and visualised, using a fluorescent marker, with a confocal microscope.

3.5.1 Inclusion formation

The polyclonal sheep antibody S830, raised against exon 1 of the huntingtin protein carrying 53 repeats, was used to investigate the formation of intranuclear inclusions in the perirhinal cortex of the R6/1 mouse. Sections were prepared from animals aged 1, 3 and 7 months old. Neuronal intranuclear inclusions were only observed in the 7 month old group.

Perhaps more interesting was a progressive development of a diffuse staining of mutant huntingtin within nuclei of cortical cells (table 3.5.1; figure 3.5.1). Within sections prepared from 1 month old animals, all cells showed none to low levels of staining. By three months, however, the majority of cells in layers II/III showed a dark, dispersed nuclear staining, while all other cortical layers showed no such

staining. At seven months of age, nuclear staining was seen in all cortical layers from II through to VI. Slices from age-matched littermate controls showed no nuclear staining at any age. Examples of sections prepared from transgenic animals of each age and a wild-type of seven months old is shown in figure 3.5.1.

	1 month		3 month		7 month	
	WT	R6/1	WT	R6/1	WT	R6/1
Layer I	–	–	–	–	–	–
Layer II/III	–	+	–	+++	–	+++ (inclusions)
Layer V/VI	–	–	–	–	–	+++ (inclusions)

Table 3.5.1 Mutant huntingtin staining in neuronal nuclei of the perirhinal cortex. Key: –, no nuclear staining; +, some nuclear staining; +++, dark nuclear staining; blue, wild types litter mates; red, transgenic animals. n=9(3).

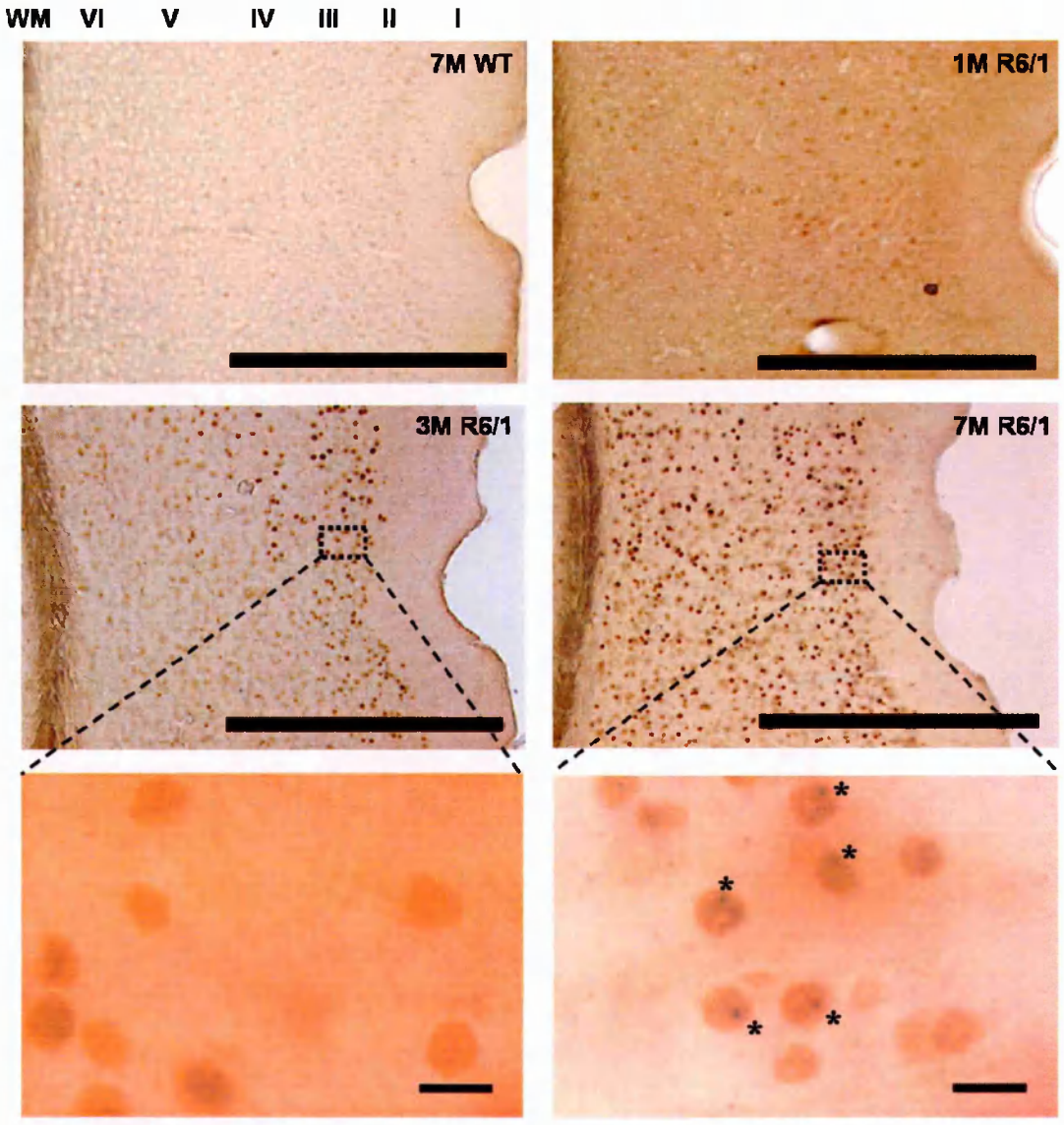


Figure 3.5.1 Progressive localisation of mutant huntingtin with the nucleus of perirhinal cortical neurones. Mutant protein was bound by an antibody raised against exon-1 huntingtin carrying 53Qs and visualised using DAB. Wild-type slices never showed any staining at any age (7 month old wild type shown here). Dark nuclear staining was not seen until 3 months of age, when layer II/III showed a disperse nuclear stain. Layer IV of area 36 also showed some staining. Aggregates were not, however, identified at this age. By 7 months, all cortical layers showed nuclear staining and some cells contained aggregates (*). Scale bar: ×100 objective images (top four): 500 μm; ×400 objective images (bottom two) 10 μm. ×400 images have been counter stained with eosin pink. Cortical layers are indicted above the 7M wild type image.

3.5.2 Dopamine receptors

Confocal microscopy was used to assess changes in dopamine receptors in the perirhinal cortex during the development of the R6/1 phenotype. Sections were prepared from the same animals used for the aggregate development study in section 3.5.1. In order to obtain fluorescence intensities for each age group, three to four animals were used per group. Images were taken from three sections prepared from each animal and composite images prepared using the Leica Confocal Software. Fluorescence measurements were obtained from each cortical layer by taking the mean of three sampling windows within each area of interest. All sections were imaged using identical confocal setting and were processed on the same day to avoid discrepancies in settings.

3.5.2.1 D_2 dopamine receptors

The expression of D_2 dopamine receptors in the perirhinal cortex were investigated in both wild-type and R6/1 transgenic mice. Preliminary studies indicated a large reduction in receptor expression in the surface layers of cortex (figure 3.5.2). Control slices, which underwent identical solution changes as experimental slices, but in the absence of either primary, secondary or both antibodies, showed very little fluorescence (figure 3.5.2). Sections prepared from D_2 knock-out mice showed little staining and, at a high PMT, no punctate staining was seen (figure 3.5.2). The D_2 dopamine receptor antibodies therefore have a high specificity.

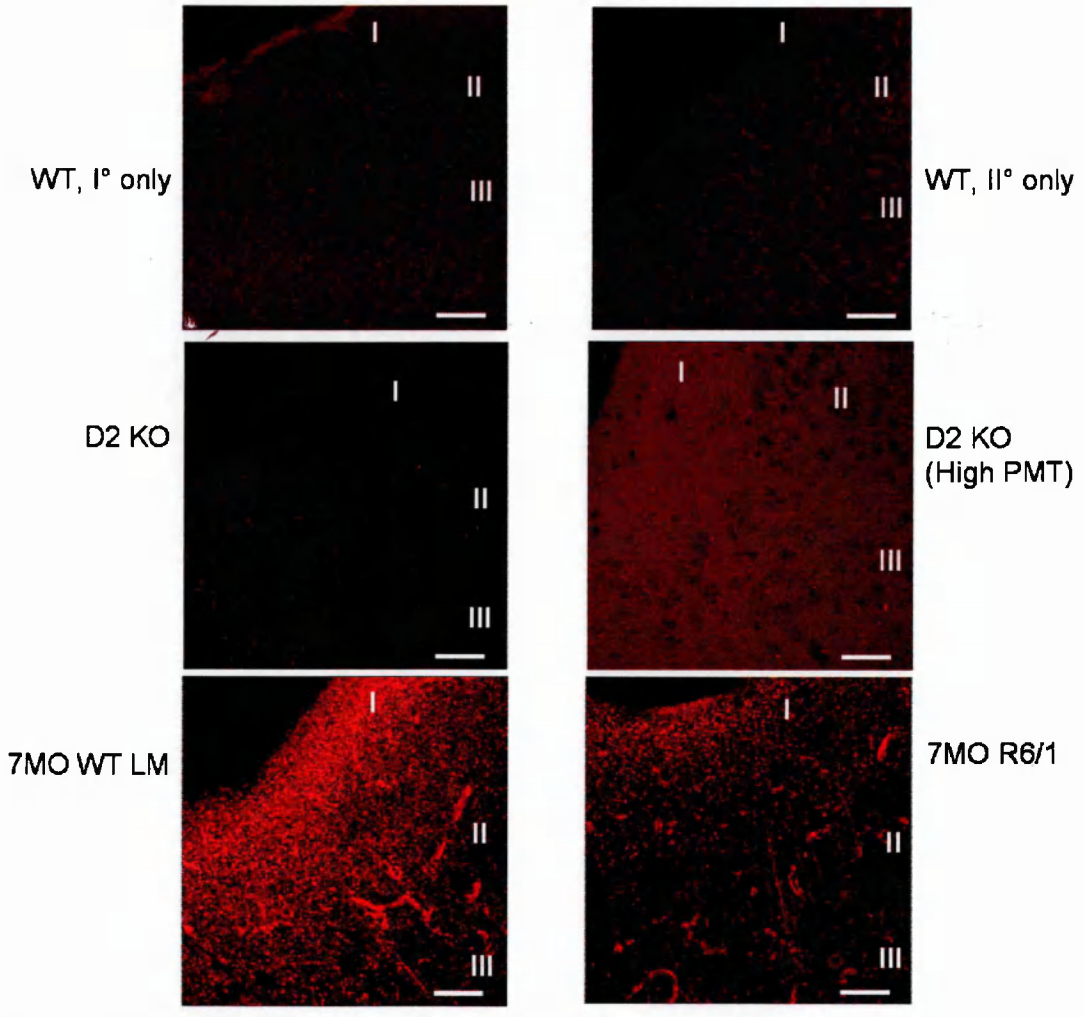


Figure 3.5.2 Control experiments for D_2 dopamine receptor immunofluorescence. *Top left* Wild type slices exposed only the primary antibodies showed only a low level of background staining. *Top right* A low level of autofluorescence was also seen when slices were exposed to secondary antibodies only. *Middle left* Slices prepared from D_2 knockout mice showed little fluorescence and no definition even at a high PMT (*middle right*). *Bottom left* Wild type slices prepared from a seven month old animal showed punctate staining predominantly in layer I. *Bottom right* Seven month old transgenic animals showed clear reduction in labelling within layer I. (N.B. All images are composite *maximal* fluorescence, while in the for analysis, confocal images are composite *mean* fluorescence.) Cortical layers are indicated. Scale bar = 100 μm. Images taken using a ×40 objective.

For analysis, three sections were prepared from three animals in each of the three age groups. Slices were prepared from the same animals as those used for the inclusion formation and the D_1 dopamine studies. Mean fluorescence was generated by taking the mean of three sampling windows in each cortical layer on images taken from each slice. Wild-type mice showed an intense band of fluorescence in layer I. During development, there was a trend towards an increase in D_2 receptor expression in all layers. This only reached significance at seven months, when all layers were significantly higher than either three or one month groups.

Layer I also showed the highest expression in the R6/1 mice. There was not, however, a developmental change in expression and intensity levels remained similar at all ages. Due to the increase seen in wild type, but not transgenic brains, there was a relative deficit of D_2 dopamine receptors in layer I transgenic perirhinal cortex at seven months (–28%; $P<0.05$; Fisher LSD test). Results are summarised in figure 3.5.3 and example images are shown in figure 3.5.4.

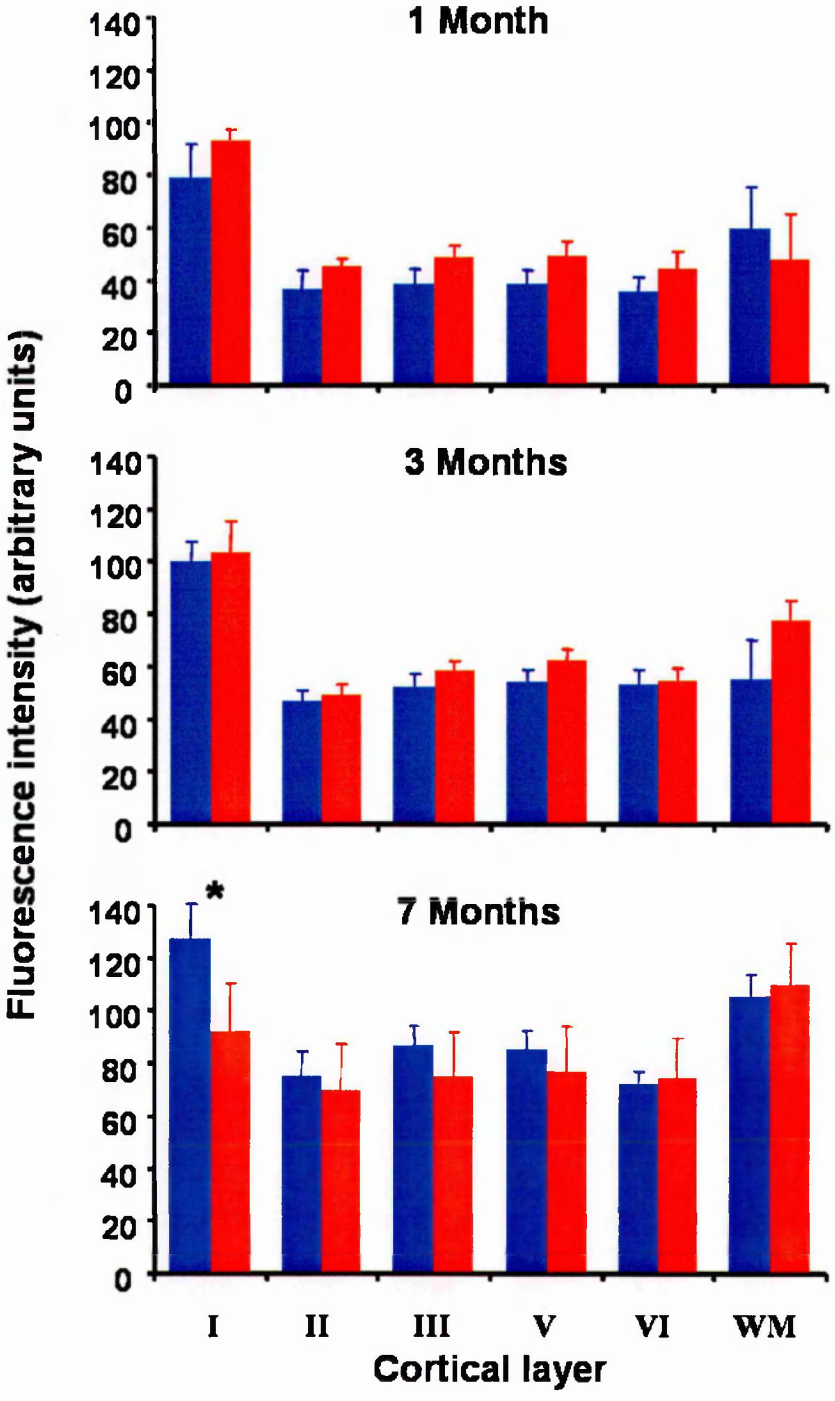


Figure 3.5.3 D_2 dopamine receptor expression in wild type and transgenic perirhinal cortex. Primary antibodies were raised against D_2 dopamine receptors and visualised using a fluorescent signal amplification system. Confocal images were obtained and intensity measurements ascertained. Will type mice (blue) showed a developmental increase in receptor expression. In contrast, R6/1 mice showed little increase with age, reflected as a reduced intensity compared to age-matched litter mates. (*= $P<0.05$ Fisher LSD test).

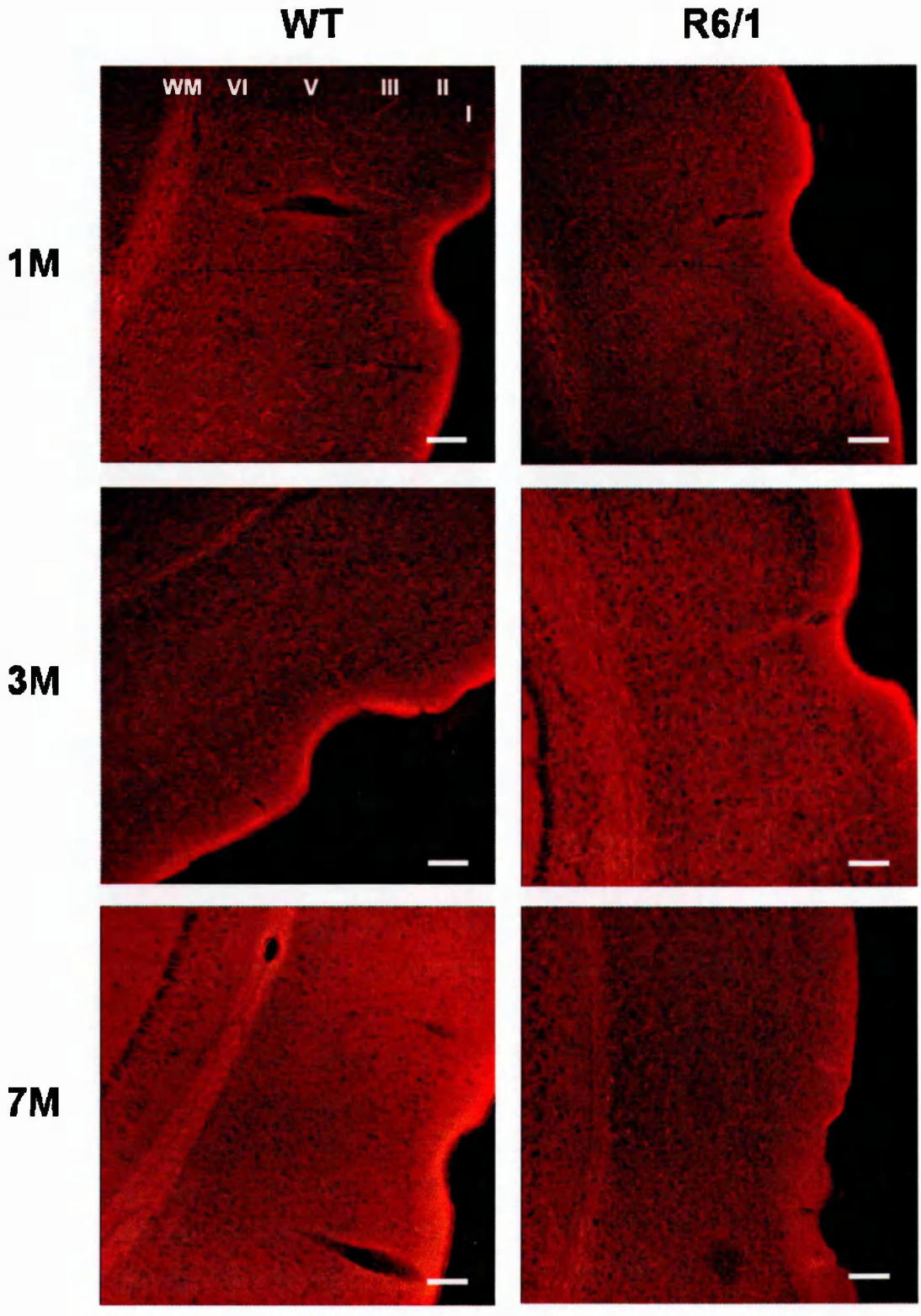


Figure 3.5.4 D_2 dopamine receptors in the perirhinal cortex. Example images obtained from both wild type and transgenic animals at each of the three age groups. The most striking difference can be seen at 7 months, where transgenic slices are clearly darker than wild types. Scale bar: 100 μm. Images obtained using a ×40 objective. Cortical layers are indicated on the top left image.

3.5.2.2 D_1 dopamine receptors

To assess D_1 dopamine receptor expression, a polyclonal antibody, raised against the D1A subclass of dopamine receptor, was used. In initial experiments, three sets of control slices, which underwent identical treatments to the experimental slices, but were not exposed to either primary, secondary or neither antibody, were prepared (figure 3.5.5). These slices showed little fluorescence when viewed under the confocal microscope. A fourth control group comprised of slices prepared from the D_2 knock-out mouse. These slices showed staining similar to slices prepared from CBA mice (figure 3.5.5). While this does not directly show specificity towards D1A receptors, it at least demonstrates discrimination from D_2 receptors.

For analysis, slices were prepared from the same animals used for the D_2 experiment in section 3.5.2.1 and the inclusion study of section 3.5.1. Again, three sections were prepared from three animals in each age group (1, 3 and 7 months). Slice mean intensity was obtained for each cortical layer and three-way ANOVA tests carried out.

Similarly to the pattern of expression of D_2 receptors in wild-type mice, D_1 showed a band of higher fluorescence in layer I than other layers at all ages. There was a developmental decrease in receptor expression in layer I (–27%; $P<0.05$, Tukey HSD post-hoc test) and a less significant reduction in layer II ($P<0.05$; Fisher LSD post-hoc test).

Fluorescence in layer I of one-month-old R6/1 mice was greatly reduced with respect to their age-matched littermates (–40%) and this reduction was highly significant ($P<0.0001$; Tukey HSD test). While the other cortical layers showed a trend towards a reduction in D_1 receptor expression, these layers failed to reach

significance. The R6/1 brains did not show a developmental down regulation in expression, although the significance in layer I was maintained at both 3 months (–22%; $P<0.02$; Fisher LSD test) and 7 months (–20%; $P<0.02$; Fisher LSD test).

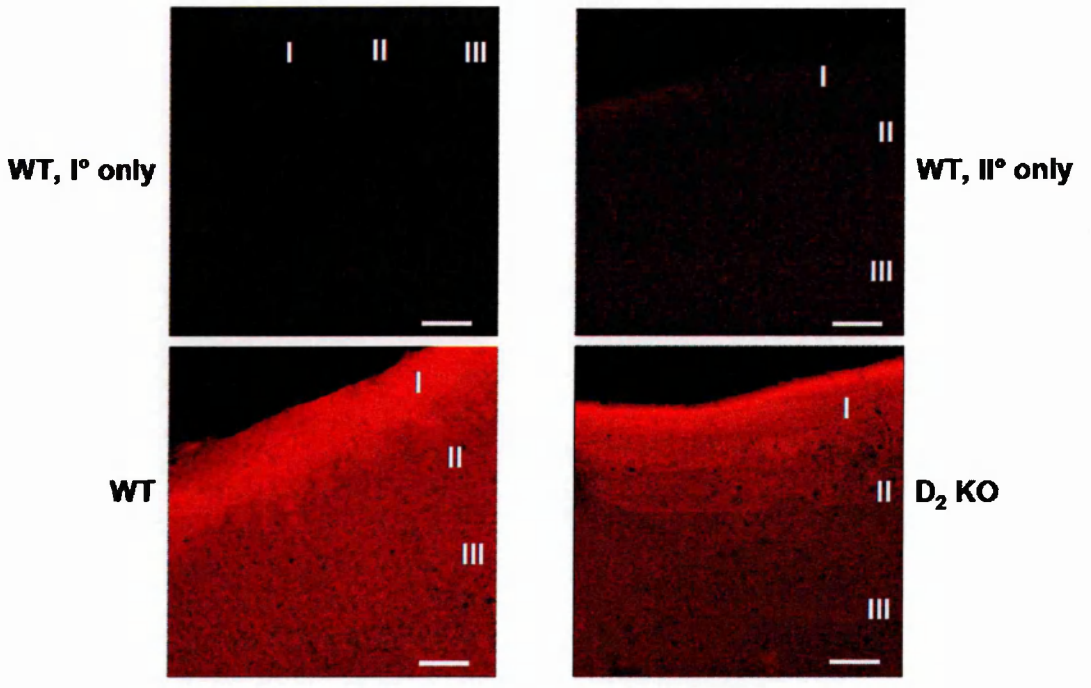


Figure 3.5.5 Control experiments for D_1 dopamine receptor immunofluorescence. *Top left* Little fluorescence is seen when wild-type slices were exposed to only the primary antibody. *Top right* Similarly, exposure to secondary antibodies only resulted in a low level of auto-fluorescence. *Bottom right* In contrast, when wild-type slices were treated fully with both antibodies, a typical pattern of fluorescence was seen. *Bottom right* When perirhinal cortical slices prepared from D_2 knockout mice were exposed to both antibodies a pattern of fluorescence similar to that seen in the wild-type mice was evident. While not being the ideal control to show antibody specificity for D_1 receptors, it demonstrates that they are not specific for D_2 dopamine receptors. The images shown here are composite images of the maximal fluorescence from a stack of 10, 1 μm images. (N.B. confocal used for analysis are composite images of the *mean* fluorescence from a stack of images.) Cortical layers are indicated. Scale bar = 100 μm. Images taken with a ×40 objective.

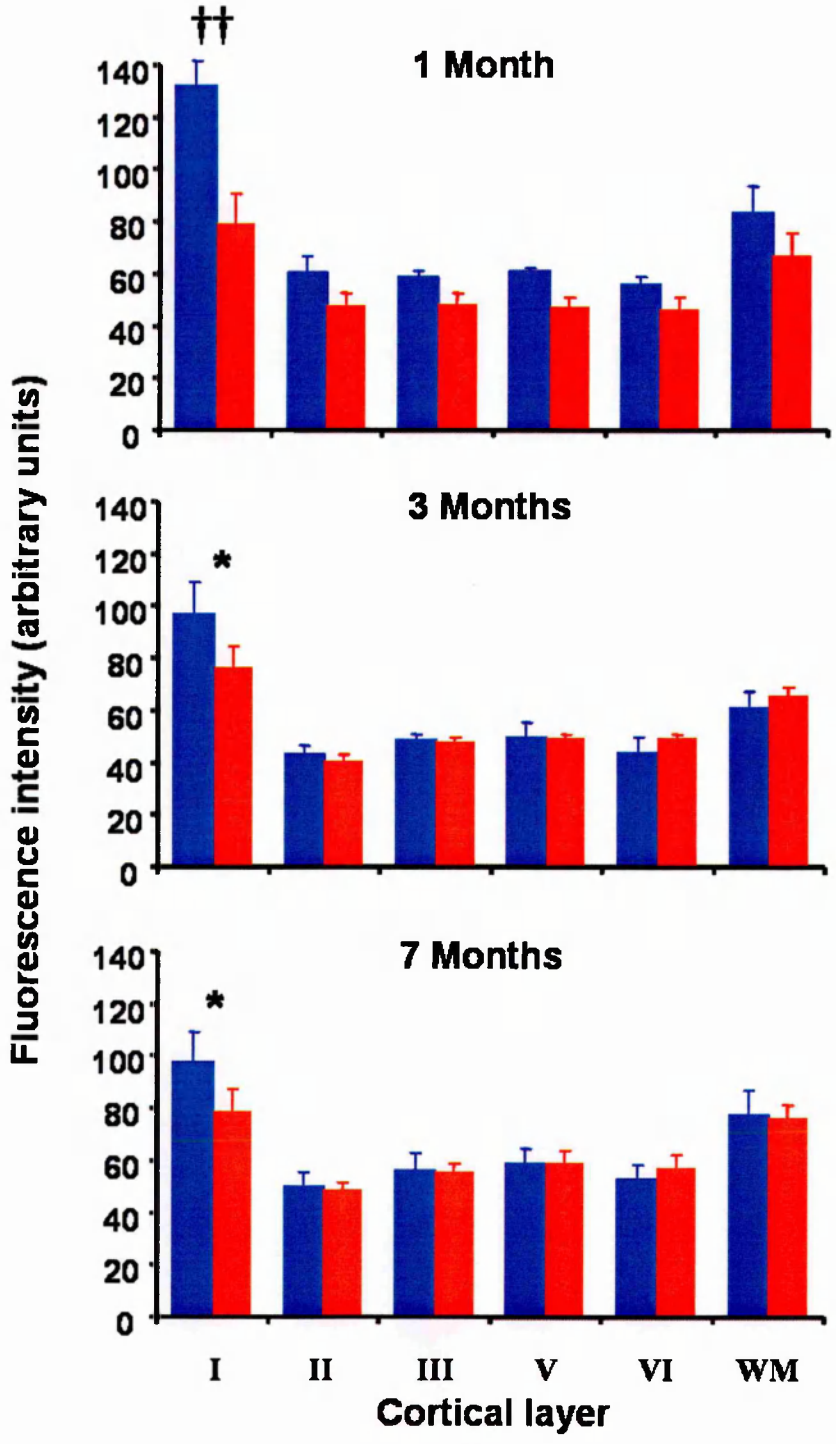


Figure 3.5.6 D_1 dopamine receptor expression in the mouse perirhinal cortex. Bound antibodies raised against D1A dopamine receptors were visualised using confocal microscopy. At one month of age, transgenic animals showed a highly significant reduction in receptors in layer I compared to age-mated littermates (††= P<0.0001 Tukey HSD test). Wild type slices showed a developmental reduction in D_1 dopamine receptor expression at 3 and 7 months of age, while transgenic slices showed no such reduction. At 3 and 7 months, D_1 dopamine receptor expression was still reduced compared to age-matched littermates (*= P<0.05 Fisher LSD test).

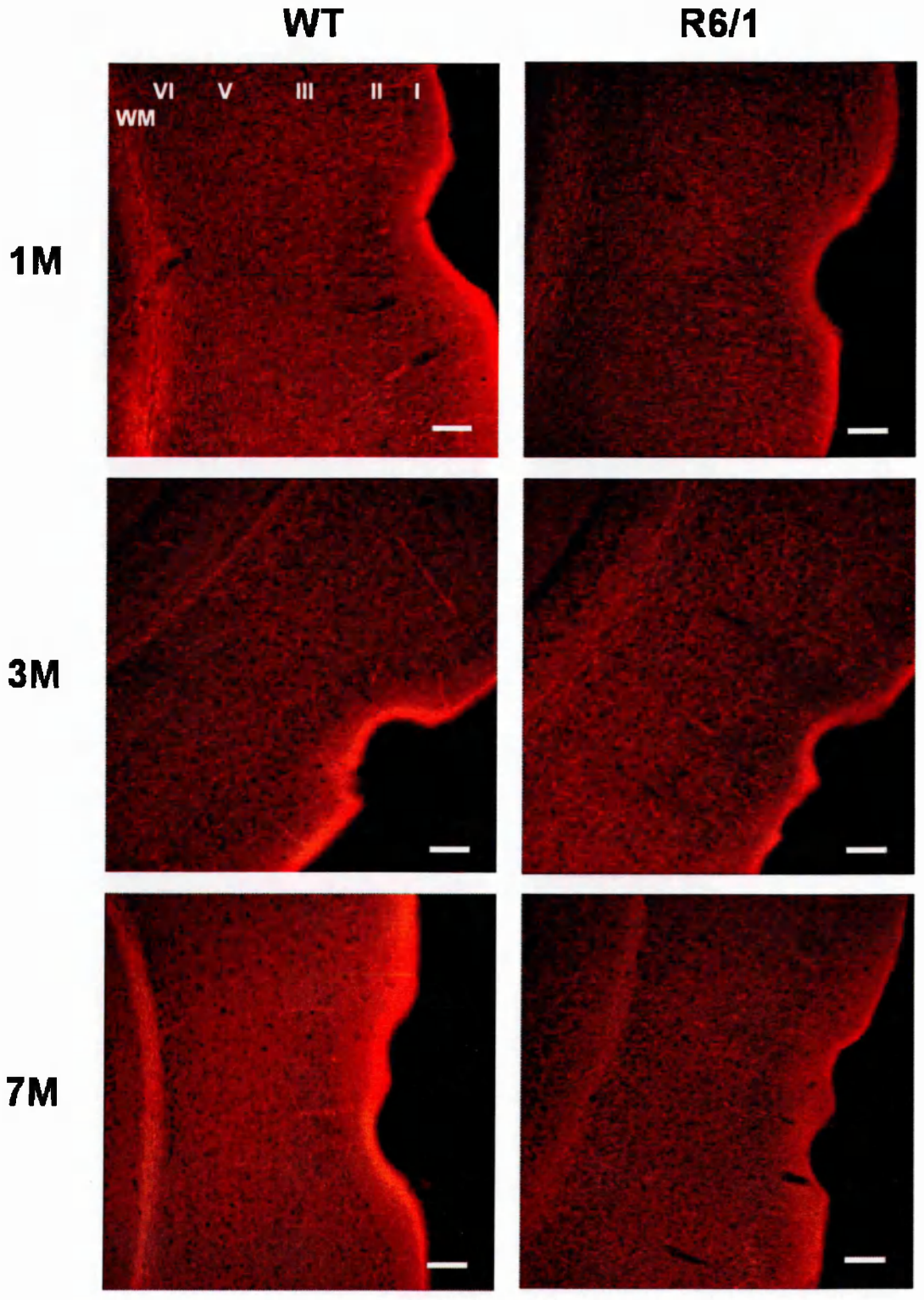


Figure 3.5.7 D_1 dopamine receptors in the perirhinal cortex. Example images obtained from both wild type and transgenic animals at each of the three age groups. Scale bar: 100 μm. Images obtained using a ×40 objective. Cortical layers are indicated on the top left image.

3.6 Summary

Perirhinal cortical synaptic plasticity in the normal mouse is predominantly that of depression, expressed as both paired-pulse depression and long-term depression. The degrees of LTD induced in slices from two though to nine months of age are comparable, as are the probabilities of induction. The age-dependency of LTD in R6/1 transgenic animals is interesting. At two months of age, LTD appears to be enhanced compare to that seem in littermate controls. The degree of depression then declines until LFS induces a slight potentiation. Remarkably, the expression of LTD could be recovered by the D_2 dopamine receptor agonist Quinpirole at both 5 and 7 months of age. Importantly, Quinpirole did not affect LTD in wild type slices.

The expression of dopamine receptors mirrors the loss of LTD. There is a progressive loss of D_1 and D_2 dopamine receptors in the surface layer of the perirhinal cortex. There are other neuropathological changes observed in the perirhinal cortex. There is an age-dependent developmental expression of mutant huntingtin within the nuclei of perirhinal cortical cells. Punctate inclusions do not appear until seven months of age, while diffuse nuclear staining is apparent in layers II/III at 3 months of age and in all layers at 7 months.

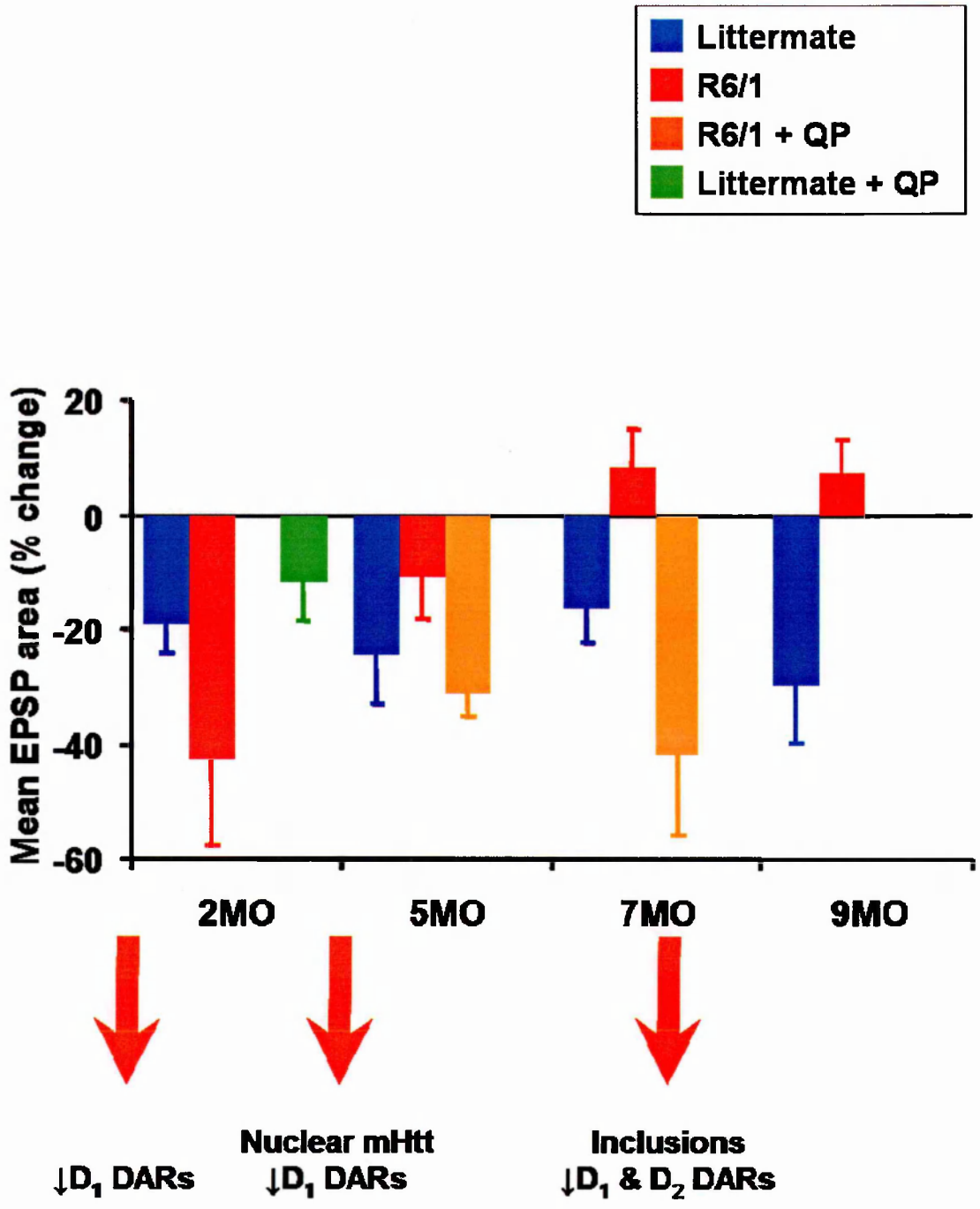


Figure 3.6.1 Key changes in synaptic plasticity at superficial synapses of R6/1 transgenic perirhinal cortex. LTD is expressed at all ages in littermates (blue). Transgenic animals (red) show enhanced LTD at 2 months and then a gradual decline, until LTD is not expressed. LTD can be recovered by applying the D_2 dopamine receptor agonist Quinpirole (orange), which does not affect LTD in wild types (green). Some of these changes may correspond to neuropathological changes observed in the R6/1 perirhinal cortex, namely loss of dopamine receptors and nuclear translocation of mutant huntingtin.

4. Discussion

4.1 Synaptic plasticity in normal mouse perirhinal cortex

This study describes both paired-pulse depression and homosynaptic long-term depression at synapses of the mouse perirhinal cortex. Both LTD and PPD have been described previously in the rat perirhinal cortex. The LTD observed in the mouse shares some properties with rat perirhinal LTD, but there are also differences. LTD induction in both rat and mouse is dependent on the activation of mGlu receptors. In contrast, NMDA receptor activation is also required for LTD induction at rat perirhinal synapses, a dependency which was not observed in the mouse. Furthermore, I have shown that long-term depression in the mouse perirhinal cortex requires the activation of dopamine D_1 and D_2 receptors; a result previously unreported.

4.1.1 Basal properties

Field potentials recorded in layer II/III in response to stimulation applied to the superficial layer I are similar in nature to responses recorded from the rat perirhinal cortex (Ziakopoulos *et al.*, 1999) and those obtained in other cortical regions (for example see Jung *et al.*, 1990; Hess & Donoghue, 1999; Sawtell *et al.*, 1999; Yun *et al.*, 2000). To enable interpretation of the field response, dual intracellular and extracellular recordings were made from layer II/III. At field responses up to ~40%

of the maximum amplitude, individual cells showed subthreshold EPSPs. At stimulus intensities that evoked field potentials greater than ~40% of the maximum, action potentials were evoked in individual cells, indicating that the field responses above this threshold are comprised of a population spike component.

Cells exhibited typical passive membrane properties of cortical pyramidal neurones, with resting membrane potential, input resistance and membrane time constant all comparable to other cortical regions (in particular see Bindman *et al.*, 1988). Action potentials were also typical of cortical pyramidal neurones, with a firing threshold of approximately −55 mV and amplitude of around 80 mV. While cell-type was not specifically confirmed using cytochemical techniques, taken together, the cellular properties suggest these cells to be pyramidal neurones.

4.1.2 Paired-pulse depression

The intermediate pathway does not appear to support paired-pulse depression at any of the intensities studied. In contrast, the superficial synapses of mouse perirhinal cortex have paired-pulse profiles that favour depression over facilitation. This is similar to the rat perirhinal cortex (Ziakopoulos *et al.*, 1999) and to other areas of neocortex (for example see Kang, 1995; Kirkwood *et al.*, 1999; Xiang *et al.*, 2002). The rat studies indicated a significant difference between the temporal and entorhinal pathways, with the temporal pathway showing greater PPD than the entorhinal pathway. Unlike the rat, no difference was seen between the two pathways in the mouse perirhinal cortex. This suggests that both pathways have equal importance in

the mouse, while in the rat, the temporal cortex may be more important than the entorhinal pathway in short-term modulation of perirhinal cortical function.

The degree of paired-pulse depression exhibited in any given pathway is always greater at higher stimulation intensities. The underlying postsynaptic cellular events may be explained by comparing both intracellularly and extracellularly recorded responses. While dual recordings were not made during paired-pulse stimulation, as discussed above, the data obtained from dual input-output relationships indicate that, from approximately 40% maximal stimulation, the field response comprises a large population-spike. It is therefore likely that the paired-pulse depression seen at paired-pulse intervals up to 1000 ms at 80% stimulation intensity is a reflection of the second shock evoking fewer action potentials than the first. Intracellularly recorded paired-pulse profiles were only obtained at stimulation intensities giving rise to subthreshold responses (corresponding approximately to intensities used to evoke a 30% maximal field response). At these intensities, intracellular paired-pulse profiles are similar to those obtained at the field level in that they are dominated by paired-pulse facilitation at intervals up to 200 ms. In light of this, it is likely that the firing of action potentials is important for PPD expression within the perirhinal cortex. Dual recordings of both field and suprathreshold intracellular potentials would reveal whether this is the case.

Paired-pulse depression is believed to have a presynaptic mechanism involving receptors located on the terminals of glutamatergic afferents. The activation of these receptors leads to the suppression of further glutamate release (Scanziani *et al.*, 1997; Vogt & Nicoll, 1999). The subclasses of glutamate receptors involved in this mechanism were investigated using receptor-specific antagonists of glutamate receptors, the results of which are summarised in figure 3.2.12. The group

II mGlu receptor antagonist had the greatest affect, altering the paired-pulse profile such that facilitation predominated. These results are consistent with presynaptic mGlu autoreceptors inhibiting further glutamate release. Similarly, application of the group I mGlu receptor antagonist also shifted the profiles towards that of facilitation. This would suggest that group I mGlu receptors are also present presynaptically at perirhinal synapses and are involved in the regulation of neurotransmitter release. The antagonism of group III mGlu receptors had no effect on the paired-pulse profile, suggesting these receptors are not involved in the short-term regulation of neurotransmitter release within this brain region. The failure of D-AP5 to effect the paired-pulse profile demonstrates that activation of NMDA receptors is not required for the expression of PPD in the perirhinal cortex.

4.1.3 Long-term depression

The intermediate pathways of the mouse perirhinal cortex do not readily support LTD at a field level. This result is similar to those obtained in the rat which also failed to show a long-lasting depression following LFS (Ziakopoulos *et al.*, 1999). In contrast, superficial synapses of the mouse perirhinal cortex did support a long lasting and homosynaptic form of long-term depression. No differences were observed between entorhinal and temporal pathways. This is in contrast to the rat studies, which showed a greater degree of frequency dependent depression in the temporal pathways than in the entorhinal pathways (Ziakopoulos *et al.*, 1999). This may reflect a relatively greater importance for the temporal cortex over the entorhinal cortex in the rat, which may not be the case in the mouse.

Glutamate receptor subtype-dependency of LTD induction at superficial synapses of the mouse perirhinal cortex was investigated by the use of antagonists and the results are summarised in figure 3.2.12. Bath application of either a group I or a group II mGlu receptor antagonist prevented the induction of LTD, showing that LTD of superficial perirhinal synapses in the mouse is mGlu receptor-dependent. Similar results were obtained in the rat perirhinal cortex when LTD was recorded at the single cell level (Cho *et al.*, 2000).

When a group III mGlu receptor antagonist was applied to perirhinal slices, there was no effect on either the induction of LTD or the expression of paired-pulse depression. Again, this is in agreement with results obtained in the rat (Cho *et al.*, 2000). Both group II and group III mGlu receptors are classically viewed as being negatively coupled to adenyl cyclase and hence down regulate cAMP production (for review see Conn & Pin, 1997). In light of this classical view of mGlu receptor coupling, it is interesting that antagonism of the group II, but not group III mGlu receptors, affected both short-term and long-term plasticity. There is evidence, however, that the cAMP pathway may not be the only transduction cascade for group III mGlu receptors. Evidence from retinal ON bipolar cells has shown that mGluR6 may also be coupled to a cyclic GMP phosphodiesterase (Shiells & Falk, 1992) and thereby regulate cGMP. This provides an alternative pathway to which the group III mGlu receptors may be coupled in the perirhinal cortex. Alternatively, there may be limited expression of group III mGlu receptors in the perirhinal cortex. *In situ* hybridisation has shown that there is no significant expression of mGluR4 and only moderate expression of mGluR7 mRNA in rat perirhinal cortex (Ohishi *et al.*, 1995). It is therefore likely that group III mGlu receptors are not functionally important in the perirhinal cortex.

Surprisingly, the NMDA receptor antagonist D-AP5 failed to block LTD induction and therefore I conclude that LTD is not NMDA receptor-dependent at mouse perirhinal cortical synapses. This is in contrast to findings in rat perirhinal cortex, where application of LFS induced a novel form of LTD that is co-dependent on the activation of both mGlu- and NMDA- receptors (Cho *et al.*, 2000). Recent preliminary data obtained in the rat has shown that application of 5 Hz low frequency stimulation (3000 pulses) leads to an NMDA receptor-independent form of LTD in neonatal and adult perirhinal cortex (Jo *et al.*, 2003). Both the results in mouse and those obtained in the rat demonstrate that the perirhinal cortex is capable of supporting different forms of synaptic plasticity and these may reflect the range of cognitive functions processed within the perirhinal cortex. In agreement with the NMDA receptor-dependency reported here, preliminary experiments in monkeys, in which AP5 was infused into the perirhinal cortex, failed to diminish accuracy on a visual recognition memory task (Turchi *et al.*, 2003). It would appear, therefore, that NMDA receptors may not be involved in such tasks in primates.

Whilst my data is in broad agreement with the published observations in the rat, there are nonetheless, as discussed above, some important differences. Several factors might account for these disparities. The first is species difference; rat and mouse perirhinal cortical synapses may support different forms of LTD. Secondly, the rat data are obtained from oblique (45°) slices. This study used coronal slices, as oblique slices of mouse perirhinal cortex did not yield robust synaptic responses. It is possible that this difference in preparation will produce slices with differentially preserved connectivity, which could affect the efficacy of the conditioning paradigms used.

When LFS was applied to synapses during intracellular recording, LTD of subthreshold EPSPs was not induced. In contrast, when a suprathreshold response was evoked during baseline, LFS gave rise to a depression of spike firing that lasted for more than one hour. Importantly, spike-LTD was found to be homosynaptic and dependent on synaptic activity rather than simply the cell firing during the conditioning period. This spike-depression is reminiscent of the decrease in action potential firing seen in perirhinal cortical neurones during the process of familiarisation. It is therefore possible that spike LTD underlies this process rather than LTD of synaptic transmission as previously proposed. The importance of altered firing properties of perirhinal cortical neurones was first suggested by Thomas Brown and colleagues, who showed that regular-spiking and late-spiking cells respond differently to trains of electrical stimuli: Regular-spiking cells reduced their firing rate during repetitive stimulation, while late-spiking cells increased their firing rate (see introduction figure 1.5.5, Beggs *et al.*, 2000). The long-term maintenance of such changes in firing rate was not, however, studied. To my knowledge, the data presented here is the first report of synaptically induced depression of action potential firing within a brain slice preparation. In contrast, forms of spike potentiation have been studied (for example see Bindman & Boisacq-Schephens, 1966; Seki *et al.*, 2003) In the Bindman and Boisacq-Stephens report, repetitive limb stimulation was found to induce prolonged discharges within neurones of the primary somatosensory cortex of rat that could last for over one hour. The extent and duration of this increase was dependent upon the degree of activity achieved within the postsynaptic cell during conditioning. While this form of spike potentiation was reported several years prior to the identification of LTP, it is clear that such increases in neuronal activity may underlie cortical function. In the more

recent Seki *et al.* (2003) article, LTP induction in the rat auditory cortex was associated with a slow depolarisation with two to five spike discharges, which was not seen either before conditioning or in those neurones that did not exhibit LTP. Given that increases in spike firing have been reported to occur within the cortex, it is conceivable that the converse, spike depression, also occurs in other brain regions.

Given that field LTD was studied at stimulus intensities set to evoke responses at 80% of the maximum, it is likely that the field LTD following LFS reflects a decrease in the number of cells firing and hence a decrease in the population-spike component. Some preliminary experiments were carried out to investigate LTD induced at different stimulation intensities. LFS failed to induce LTD at 30% stimulation intensity (n=3(1)), which fits with the lack of LTD recorded intracellularly at subthreshold intensities.

It should be noted that the protocols used to induce LTD at the field and intracellular levels were different. At the field level, 900 pulses applied at 1 Hz were used to induce LTD, while LFS applied during intracellular recordings consisted of a shortened period of shocks (300 pulses at 1 Hz). This difference was required because the longer LFS paradigm led to the loss of the intracellular recording. This may simply be a consequence of the sensitivity of cellular impalement. Conversely, it may reflect cellular death during conditioning. If this were the case, the LTD recorded at the field level would be the corollary of many cells dying during the conditioning period and hence less ionic currents underlying the field response. However, given that the shorter period of LFS gives rise to spike-depression and not cell death, the later is not likely to be the underlying cause of the field LTD. In contrast, preliminary field experiments in which 300 pulses were used to induce LTD failed to demonstrate depression of the field potential (n=3(1)). This is not, however,

necessarily an indication that the prolonged 900 pulse-LFS causes cell death. The field LTD induced by 900 pulse-LFS may be a reflection of multiple cells displaying spike-LTD, while 300 pulse-LFS may induce spike-depression in only a few cells. Thus, the ionic currents resulting from spike depression induced by the shorter conditioning period may be masked by the many cells that still fire action potentials. It is also possible, given LTD is only seen 60% of the time in the original control experiments, that these preliminary experiments fall into the 40% that do not show LTD.

It would be interesting to record intracellularly from multiple cells, both proximal and distal to the field electrode, in order to estimate the number of neurones that undergo spike-depression during LTD.

4.1.4 GABA receptors

The inhibitory activity of GABA receptors is important for the expression of both short-term and long-term plasticity in the mouse perirhinal cortex. The role of $GABA_A$ receptors is complicated. Antagonists of $GABA_A$ receptors enhanced the degree of depression seen at any given paired-pulse interval. This is perhaps contrary to what one may predict as the effect of removing the inhibitory activity of $GABA_A$ receptors. As reported previously, blockade of $GABA_A$ receptors within the geniculo-cortical visual pathway of rat prevents the expression of PPD (Jia *et al.*, 2004). A possible explanation of the result obtained in the perirhinal cortex could be that paired-pulse depression in this region is not mediated by $GABA_A$ receptors *per se*, but rather PPD is a glutamatergic phenomenon in which $GABA_A$ activity

regulates the excitability of afferents within the perirhinal cortex. With the removal of the regulatory role of $GABA_A$ receptors, more glutamate may be released from the presynaptic terminal, thereby activating more autoreceptors and hence greater PPD. Alternatively, the location of the $GABA_A$ receptors could be on the dopaminergic terminals. In this scheme, the normal dopaminergic transmission that is required for PPD in this brain region is increased and hence the expression of PPD enhanced.

Blockade of $GABA_A$ receptors prevented the induction of LTD. The profile of the conditioning period reflected the altered paired-pulse profile in that a large degree of depression was achieved immediately following onset of LFS. Responses returned to baseline levels immediately following conditioning. This would suggest that LTD of the glutamatergic response could be a reflection of LTP in the GABAergic pathways of the perirhinal cortex. While this mechanism has not previously been reported for LTD, the converse, disinhibition (LTD of GABAergic inputs giving rise to LTP), has been demonstrated in the rat CA1 field (McMahon & Kauer, 1997). It should also be taken into consideration that neurosteroids, levels of which maybe elevated during the stress of slice preparation, have a potentiating effect upon $GABA_A$ receptors by increasing chloride currents (Puia *et al.*, 1990). It is therefore possible that $GABA_A$ inhibitory currents maybe more susceptible to potentiation as an artefact of stress responses.

$GABA_B$ receptors are also involved in short-term plasticity in the mouse perirhinal cortex. Application of a $GABA_B$ receptor antagonist (SCH50911) prevented the expression of PPD at intervals between 100 and 1000 ms, but not at intervals less than 100 ms. This result is similar to that obtained in the rat, where the role of $GABA_B$ receptors was first suggested by the paired-pulse profile which has a maximum depression at 200 ms (the typical time course of metabotropic $GABA_B$

receptor activity). The role of $GABA_B$ receptors was then confirmed using two different $GABA_B$ receptor specific drugs (3-*N*[1-(S)-(3, 4-dichlorophenyl)ethyl]amino-2-(S)-hydroxypropyl-p-benzyl-phosphinic acid (CGP55845A) or 3-amino-propyl(diethoxymethyl)phosphinic acid (CGP35348)), both of which blocked PPD at the intervals between 100 and 1000 ms (Ziakopoulos *et al.*, 1999; 2000; Garden *et al.*, 2002). Given that $GABA_B$ receptors are coupled to inwardly rectifying potassium channels (see Marshall *et al.*, 1999 for review) and that blockade of $GABA_B$ receptors block PPD, one would predict that inward rectifying potassium channels may underlie PPD. However, in the rat, blockade of these receptors using barium does not prevent the expression of PPD (Ziakopoulos *et al.*, 2000), suggesting a mechanism other than inward potassium rectification in perirhinal cortical PPD. One such mechanism has been recently highlighted in the rat CNS in which presynaptic $GABA_B$ receptors are negatively coupled to adenyl cyclase and thus down regulate PKA (Kubota *et al.*, 2003). Activation of PKA is known to enhance currents through L-type voltage sensitive calcium currents (Catterall, 2000) and therefore suppression of PKA by $GABA_B$ receptor activation could underlie PPD in the perirhinal cortex. The PKC pathway may also be linked to $GABA_B$ receptors and may exert an effect on transmitter release either directly (Kamatchi & Ticku, 1990) or indirectly through cross-talk with the PKA pathway (Kubota *et al.*, 2003).

The $GABA_B$ receptor antagonist also precluded the induction of LTD. The degree of frequency-dependent depression achieved during conditioning was reduced. Immediately following LFS, responses returned to baseline, but then showed a slow-onset depression that returned to baseline within one hour. It therefore appears that $GABA_B$ receptors are also important for the induction of LTD.

4.1.5 Dopamine receptors

In addition to the involvement of glutamate and GABA receptors in synaptic plasticity, the involvement of D_1 and D_2 dopamine receptors was also examined. The cerebral cortex receives dopaminergic inputs that originate principally in the ventral midbrain tegmentum (Lindvall *et al.*, 1974). Consistent with this, D_1 and D_2 dopamine receptors have been identified in the perirhinal cortex where they are reported to be located predominantly in the superficial layers I and II and also the deep layers V and VI (Richfield *et al.*, 1989). Despite their identification, there has previously been no electrophysiological demonstration of dopamine receptor function in the perirhinal cortex.

The results presented in this thesis provide evidence that dopaminergic modulation, with respect to D_1 and D_2 receptors, affects both short-term and long-term synaptic plasticity of glutamatergic transmission in the perirhinal cortex. The classical view of dopamine transduction is that D_1 and D_2 receptors are positively and negatively coupled to the cAMP cascade respectively (Stoof & Kebabian, 1981). In terms of their distribution, it is generally assumed that they follow that of other brain regions, that is D_1 receptors are predominantly postsynaptic whereas D_2 receptors are found either side of the synapse (for review of dopamine receptors see Jaber *et al.*(1996)). It is interesting, therefore, that blockade of either D_1 or D_2 receptors prevented the induction of LTD, suggesting that the role of dopamine in perirhinal LTD may be contrary to a traditional interpretation.

In an attempt to resolve this apparent disparity both D_1 and D_2 receptor antagonists were co-applied. Curiously, the co-application of the two dopamine receptor antagonists resulted in the full recovery of PPD at superficial synapses.

Furthermore, there was a partial recovery in the ability to express, though this failed to achieve statistical significance. The partial recovery of the inductive mechanism for LTD in the presence of both D_1 and D_2 receptor antagonists suggests that the role of dopamine in the perirhinal cortex is complex.

One possible explanation could be that an appropriate balance between the two opposing roles of the dopamine receptor subclasses is needed for LTD expression. Separate blockade of either subclass of receptor would therefore lead to failure to express plasticity, while preventing activation of both receptors restores the balance of D_1:D_2 receptor activity, resulting in the expression of PPD and LTD. The lack of significance of the LTD result in the presence of both antagonists may be an artefact of varying affinities for, or potencies at, the respective dopamine receptors of the two drugs. If the correct concentrations were used to reinstate the normal balance between the subclasses, LTD that is identical to controls may have been induced.

Another factor that may be important in determining the role of dopamine receptors is their synaptic localisation. Given the assumption that paired-pulse depression is a presynaptic phenomenon, the fact that D_2 and, to a degree, D_1 dopamine receptors alter the paired-pulse profiles, indicates that these receptors are heterosynaptic receptors located on the presynaptic glutamatergic terminal, where they regulate the release of glutamate. On the other hand, the experiments reported in section 3.2.2.4, in which application of the D_2 receptor agonist leads to hyperpolarisation of the cell membrane and an associated reduction of input resistance, indicates a postsynaptic location of this subclass of receptor. In contrast, application of a D_1 dopamine agonist had little effect on either membrane potential or input resistance. This would indicate that D_1 dopamine receptors have limited postsynaptic expression in the perirhinal cortex. The synaptic distribution of

dopamine receptors has not been studied extensively in the cerebral cortex. Electron microscopic studies within prefrontal cortex indicates both a pre and postsynaptic localisation of D_2 (Wang & Pickel, 2002) and D_1 (Bergson *et al.*, 1995) receptors. In the striatum, similar experiments reveal that both subtypes of dopamine receptor are located predominantly postsynaptically, with both receptors also showing some presynaptic expression (Levey *et al.*, 1993). There is no reason, however, to assume that the distribution within the perirhinal cortex should have the same receptor distribution as that of the prefrontal cortex, the striatum or any other brain region.

4.1.6 Receptor transduction pathways

There is an intriguing link between the synaptic distribution of the receptor classes, their down-stream messenger cascades and the receptor antagonism that produced the most overt affects on paired-pulse depression. Antagonists of the D_1 dopamine- and group I mGlu- receptors produced only modest shifts in the paired-pulse profiles. Both of these receptor classes are reported as having a predominantly postsynaptic location (Bergson *et al.*, 1995; Lopez-Bendito *et al.*, 2002). (Although a predominantly presynaptic locale cannot be ruled out, as antagonists of both receptors altered PPD and, as discussed above, D_1 agonism does not have a large postsynaptic effect.) In contrast, antagonism of the D_2 and the group II mGlu receptors produced highly significant changes in paired-pulse outcome and blocked the induction of LTD. These two receptor classes have a presynaptic distribution, with D_2 also located postsynaptically (Wang & Pickel, 2002; Petralia *et al.*, 1996). Furthermore, both of the receptors are negatively coupled to adenyl cyclase and

down-regulate cAMP production (Stoof & Kebabian, 1981; Conn & Pin, 1997 (review)). Therefore, both pre- and postsynaptic decreases in cAMP production appear to play an important role in both short-term and long-term synaptic plasticity in the perirhinal cortex.

Another transduction cascade that maybe important in perirhinal LTD maybe the MAP kinase pathway. In the prefrontal cortex, the four receptor classes that have the largest effect on perirhinal cortical plasticity, have been proposed to be coupled to the mitogen activated protein kinase (MAPK) pathway, where they converge postsynaptically to induce LTD (Otani *et al.*, 1999). Preliminary experiments using a MAPK-kinase inhibitor 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one (PD98059, Alessi *et al.*, 1995; Dudley *et al.*, 1995) were carried out in the mouse perirhinal cortex to investigate this potential pathway. While the drug appeared to preclude LTD induction, the results are difficult to interpret reliably. This drug is not water soluble and required dimethylsulphoxide (DMSO) as a solvent. Unfortunately, DMSO itself had adverse long-term affects on slice integrity, leading to a decline in the quality of responses. To ensure comparability between experiments, slices were incubated in DMSO prior to experimentation and at least one hour allowed after the addition of PD98059 for the drug to equilibrate. Despite these complications, it appears that the MAPK-kinase inhibitor does impede the induction of LTD above that of the vehicle alone experiments (figure 4.1.2). LTD in the presence of DMSO was significant at one hour post conditioning (−15.2±5.4%; n=5(3); $P<0.05$, two-tailed, paired t-test). Moreover, PD98059 blocked the induction of LTD (−1.2±4.9%; n=5(4)) and this was significantly different at the 10% level compared to the vehicle condition ($P=0.09$; two-tailed, unpaired t-test). With a larger n value, I predict this would reach significance at the 5% level.

Activation of the MAPK pathway is associated with an increase in protein synthesis of the early immediate gene *zif268* which is known to play important roles in both long-term potentiation and memory formation (Spektor *et al.*, 2002; Bozon *et al.*, 2003a; 2003b). Down regulation of the MAPK pathway through the activation of either D_2 dopamine or group II metabotropic glutamate receptors is therefore proposed to bring about LTD though a reduction in postsynaptic protein synthesis.

The mechanisms highlighted by this study that appear to be important in perirhinal cortical synaptic plasticity are summarised in figure 4.1.3.

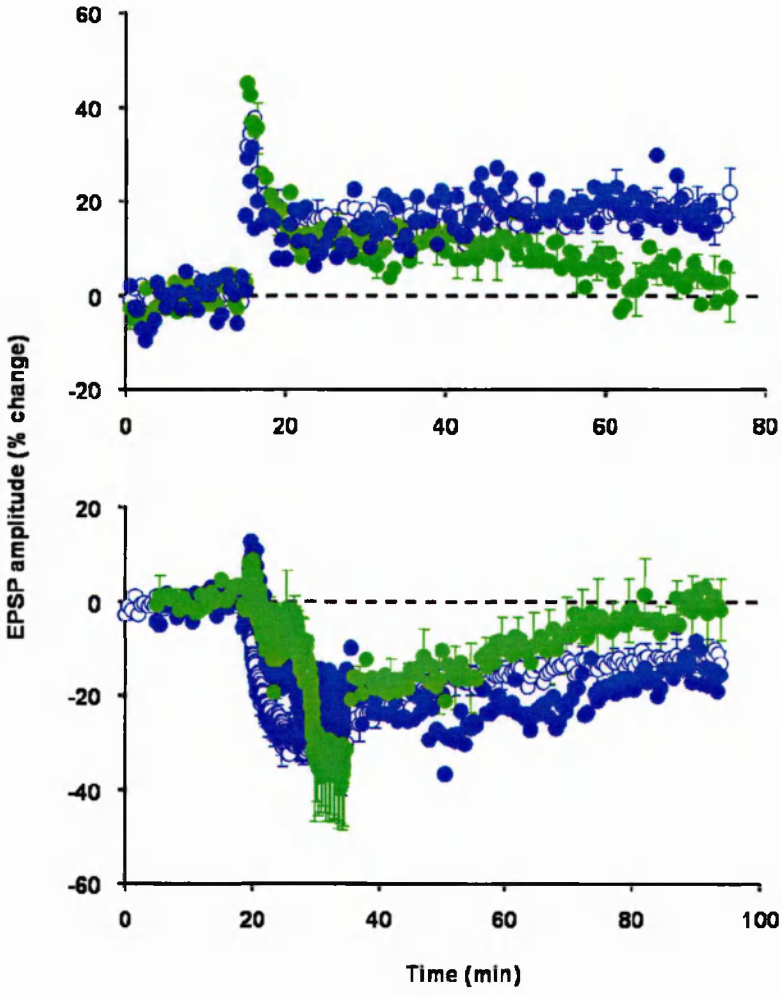


Figure 4.1.2 Dependency of long-term synaptic plasticity on the MAP kinase transduction cascade. *Top* The MAP kinase kinase inhibitor, PD98059 (●; 10 μM) prevented the expression of LTP. The drug solvent DMSO (●) did not significantly alter LTP induction from that seen in control experiments (○). *Bottom* PD98059 also blocked the expression of LTD above that of DMSO.

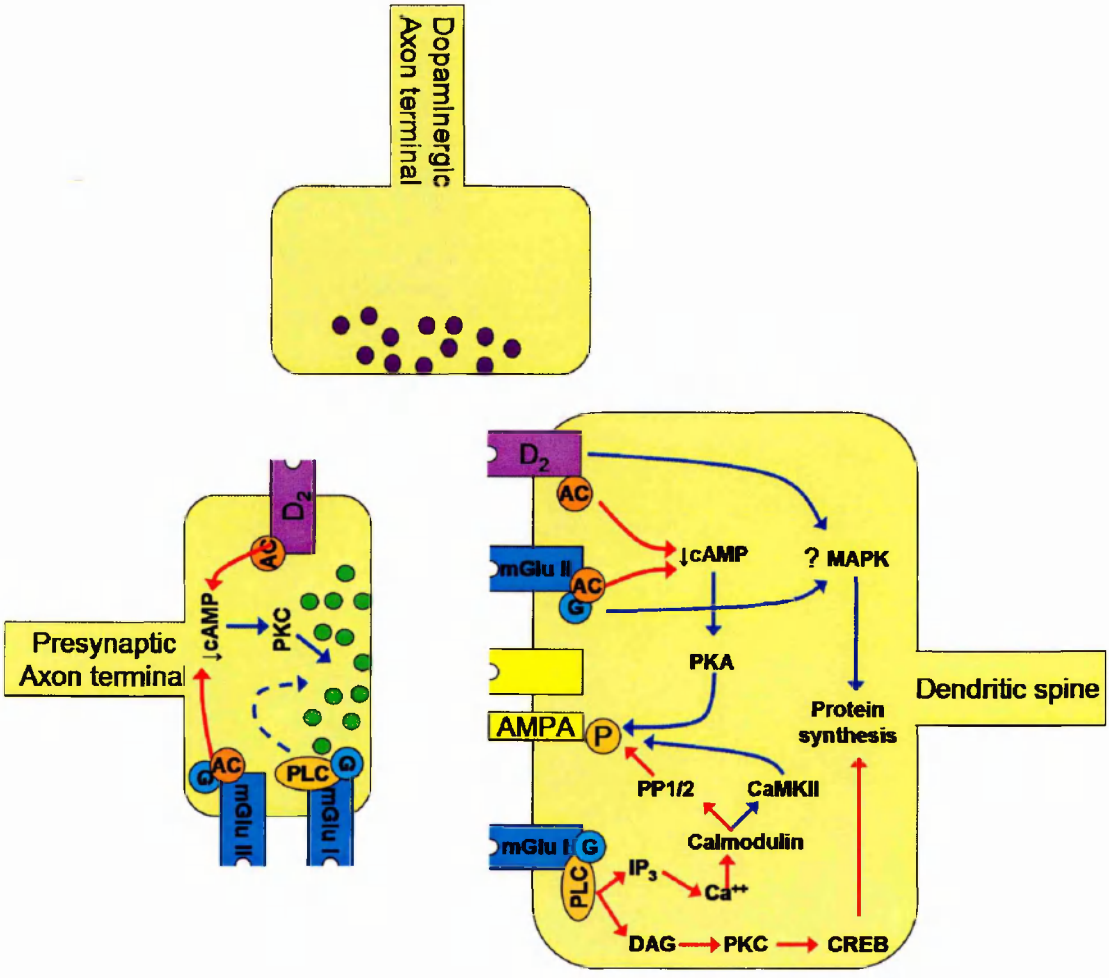


Figure 4.1.3 Signal transduction in the perirhinal cortex. Short-term modulation of the release of glutamate (●) from the presynaptic terminal is mediated by both group I and group II mGlu autoreceptors. An important modulator of glutamate release is also dopamine (●) acting on D_2 heteroreceptors located on the presynaptic terminal. Postsynaptic receptors important in the expression of long-term synaptic plasticity are again groups I and II mGlu receptors and D_2 dopamine receptors. The common transduction cascades of group II mGlu and D_2 dopamine receptors include the down regulation of cAMP and possibly MAPK. Activation of the group I mGlu receptor subclass leads to both IP_3 and DAG production and hence the activation of their subsequent cascades. Red arrows indicate pathways thought to be activated during perirhinal cortical LTD. Blue arrows indicate down regulated pathways.

4.1.7 Is LTD in the perirhinal cortex presynaptic?

A consistent observation throughout this study is that drugs that alter the paired-pulse profile from depression to facilitation also block the induction of LTD. This may suggest that LTD in the perirhinal cortex has a presynaptic locus of expression. Preliminary experiments aimed at resolving the locus of expression did not yield consistent results. Paired-pulse ratios were monitored before and after LTD induction; such experiments have previously been used to determine presynaptic changes in the probability of release during synaptic plasticity (for example see McNaughton, 1982; Kuhnt & Voronin, 1994; Schulz *et al.*, 1994; Kleschevnikov *et al.*, 1997). In the perirhinal cortex, some experiments showed an altered paired-pulse ratio, which is consistent with a decreased probability of release following conditioning. Other experiments showed no change in paired pulse depression and these later results are consistent with a postsynaptic locus of expression. It is possible that these inconsistencies are a reflection of different forms of LTD expressed within the perirhinal cortex.

The link between the expression of paired-pulse depression and LTD expression in each drug condition is interesting. This relationship between short and long-term plasticities has also been reported at corticostriatal synapses (Akopian *et al.*, 2000). At those corticostriatal synapses that displayed PPD, tetanic stimulation gave rise to LTD. Conversely, those synapses displaying PPF exhibited LTP following tetanus. Similar relationships have been reported in the hippocampus (Son & Carpenter, 1996) and neocortex (Volgushev *et al.*, 1997). In both of these regions, high-frequency stimulation gave rise to LTP at synapses displaying large PPF, while

inducing LTD at synapses with a small PPF ratio. These data suggest the degree of presynaptic calcium is an important determinant in the direction of plasticity observed. These results are consistent with those obtained here in the perirhinal cortex, suggesting that perirhinal LTD may have a presynaptic component.

4.1.8 Long-term potentiation

The superficial pathway of the mouse perirhinal cortex readily supports LTP. This is very similar to the rat perirhinal cortex, which supports LTP of the field response in both the horizontal (Bilkey, 1996) and oblique (Ziakopoulos *et al.*, 1999) slice preparations. In contrast to both of these independent reports, LTP is not NMDA receptor-dependent in the mouse. This discrepancy may be due to the differences in preparation from both the rat studies. However, given the consistency in NMDA receptor dependency between the two rat preparations, it seems more likely that rat and mouse perirhinal cortex support different forms of LTP.

Blockade of either group I or group II metabotropic glutamate receptors does not affect LTP induction. The group III antagonist showed a trend to reduce the degree of LTP, but this failed to reach significance. The mGlu receptor-dependency of perirhinal cortical LTP has not previously been reported in any species. Given that in many brain regions LTP is often dependent on either NMDA or mGlu receptors, it is interesting that neither NMDA nor mGlu receptors are involved in the induction of LTP in the perirhinal cortex. It would be interesting to investigate the role of VSCC in perirhinal cortical LTP induction. Blockade of VSCCs have

previously been reported to prevent the induction of a calcium-dependent/NMDA receptor-independent form of LTP in CA1 hippocampal neurones.

Despite the apparent independence of LTP on glutamate receptors, there appears to be a role for dopaminergic receptors. While antagonists of dopamine receptors had little effect on LTP, an agonist of D_1 dopamine receptors precluded LTP induction and an agonist of D_2 dopamine receptors enhanced LTP. Taken together with the paired-pulse and LTD studies, it is clear that dopamine is an important neuromodulator within the perirhinal cortex.

Given that dopamine appears to modulate long-term changes in glutamatergic transmission in the perirhinal cortex, it is reasonable to suggest that cAMP and PKA are mediators of LTP in this brain region. While experiments designed to study this have not been carried out, experiments to elucidate some of the transduction mechanisms involved have. The MAP kinase kinase inhibitor PD98059 was applied to slices and found to prevent the expression of LTP, but not short-term potentiation. In vehicle alone (DMSO), LTP was expressed similarly to control experiments (18.0±1.8%; n=4(3)). In the presence of the drug, the EPSP amplitude returned to baseline by one hour (3.6±4.7%; n=9(5)). The difference was significant only at the 10% level (P=0.075; two-tailed, unpaired t-test). Similarly to the LTD experiments in the presence of this drug, larger n values would probably increase the significance of this result. These results suggest the MAP kinase pathway is involved in the expression of LTP in the perirhinal cortex.

A physiological role of LTP in the perirhinal cortex has yet to be suggested, but it is likely to underlie one or more of the associative cognitive processes known to be processed within the perirhinal cortex (for instance fear conditioning or stimulus-stimulus association). It would be interesting to carry out experiments to

test if LTP in the perirhinal cortex is mediated by a pure increase in the EPSP slope, or, as with LTD, a change in action potential firing and hence a change in the field pop-spike component.

4.2 Synaptic plasticity in the R6/1 perirhinal cortex

The R6/1 perirhinal cortex shows a progressive abnormal electrophysiological phenotype. The development of the synaptic phenotype does not follow the onset of the overt motor phenotype. Several months prior to the onset of clasping and notable weight loss, aspects of the normal synaptic phenotype are enhanced. As the transgenic phenotype starts to become apparent (at approximately five months), both perirhinal neurones and synapses start to show declines in normal function compared to wild types.

4.2.1 Cellular properties

Perirhinal cortical neurones undergo distinct changes in their basal properties during the lifetime of the R6/1 mouse. At three months of age, perirhinal neurones appear to be normal with regards resting membrane potential, input resistance, membrane time constant, cell capacitance and action potential properties, including threshold, size and duration. At five months, two populations of cells were identified: One with normal cellular properties; and another with a depolarised resting membrane potential. By 7 months, the majority of neurones are depolarised and show a

significantly reduced membrane capacitance. While hippocampal CA1 pyramidal cells of the R6/2 mouse were reported as normal with regards membrane potential (Murphy *et al.*, 2000), MSSNs of the striatum do show a similar depolarisation (Levine *et al.*, 1999). Similarly, a subpopulation of MSSNs in a knock-in mouse carrying 94 CAG repeats show a greater membrane depolarisation than MSSNs from a CAG71 knock-in mouse (Levine *et al.*, 1999).

The depolarised resting membrane potential is likely to be a result of impaired energy metabolism, which has been suggested to occur in HD. Evidence for energy impairment comes from the systemic administration of mitochondrial inhibitors (see introduction, section 1.2.7.1) which can recapitulate an HD-like phenotype in rats and primates. The resultant deficiency in ATP availability would interfere with Na^+/K^+/ATPase activity, which is necessary for the maintenance of intracellular concentrations of potassium and sodium ions, the principle determinants of membrane potential. Alternative or additional explanations of a depolarised resting membrane potential could be an increased level of extracellular glutamate, a result of impaired glutamate reuptake. Impaired glutamate uptake has been reported in both the cortex and striatum of the R6/2 mouse as a result of decreased expression of the GLT1 glutamate transporter (Liévens *et al.*, 2001). Yet another explanation could be the enhanced function of NR2B receptors associated with mutant huntingtin (Chen *et al.*, 1999b). Enhanced currents though these receptors could lead to a prolonged depolarisation and thus contribute to the abnormal resting membrane potential seen in cortical (and striatal) neurones. If this last explanation is true, this sets up a positive feedback loop whereby enhanced NMDA receptor function leads to depolarisation, which, in turn, contributes to prolonged activity of the NMDA receptor itself.

A reduced membrane capacitance is consistent with a reduction in cell size. The specific membrane capacitance of all biological membranes is approximately 10 nF/mm^2 (Kandel *et al.*, 2000). Applying this constant to the data obtained here gives an approximate surface area of normal layer II/III pyramidal cells within the mouse perirhinal cortex of 0.02 mm^2. Similarly, in young transgenic animals, the layer II/III pyramidal neurones have an estimated surface area of 0.02 mm^2. Neurones in slices from older transgenic animals (7 months) were reduced in size and have an approximate surface area of 0.01 mm^2. Such a decrease may be a result of dystrophic neurites and neurite retraction, which has been reported in human HD cerebral cortex (for example see Jackson *et al.*, 1995; DiFiglia *et al.*, 1997). A decrease in cell size may account for the decrease in brain weight in the absence of significant cell death that is observed in the R6 mouse. The reduction in cell size may have led to a closer packing of cells and this could explain the observation that the transgenic neurones were generally easier to find using the blind techniques used in this study. It is also possible that there is a reduction in the size of somatic cells throughout the whole body and this may account for the loss of body mass observed in R6 mice.

The abnormal action potential properties identified in transgenic slices may be attributable to the depolarised resting membrane potential and its proposed underlying cause, energy impairment. With a reduced electrochemical gradient, the sodium ion flux underlying the action potential will be less, giving rise to a smaller, broader action potential. The other notable change in action potential firing was a decrease in the number of spikes fired in response to a depolarising current step. Impairments in the ability to sustain trains of action potentials may have profound implications on information processing within the perirhinal cortex.

4.2.2 Field responses in transgenic slices

Extracellularly recorded field potentials were also notably different, characterised by a large, late component. This slow component appeared similar to that seen when a $GABA_A$ receptor antagonist is applied to wild type slices, exposing NMDA-mediated currents. Application of an NMDA receptor antagonist, however, did not eliminate the late component, suggesting other mediators than NMDA channels. The role of $GABA_A$ receptors were investigated further by applying PTX to the slices at the same concentration as that used for intracellular experiments in control slices. PTX did not, however, alter the late component. While higher concentrations of PTX were not applied to slices, it is not likely that $GABA_A$ receptors underlie this component. Evidence for this comes from the disparity between the input-output relationships, paired-pulse profiles and LTD profiles obtained from transgenic animals compared to those recorded in wild type slices in the presence of a $GABA_A$ antagonist (compare figure 3.3.5 and figure 3.2.9). Similarly, it is not likely that $GABA_B$ mediated currents underlie the late component; again, the input-output relationships, paired-pulse profiles and LTD profiles of transgenic and wild type slices in the presence of a $GABA_B$ receptor antagonist do not match (see figure 3.3.5 and figure 3.2.10).

Despite the recovery of normal synaptic plasticity in transgenic slices by a D_2 receptor agonist, Quinpirole did not abolish the late component. In fact, the late component appeared totally resistant to pharmacological manipulation. Both agonists and antagonists of both D_2 and D_1 dopamine receptors also failed to abolish this late component, as did an agonist of group II mGlu receptors. The underlying cause of the late component therefore remains unresolved.

4.2.3 Paired-pulse stimulation

Short-term synaptic plasticity was affected in the transgenic slices compared to age-matched littermates. At two and five months of age, normal paired-pulse depression was exhibited at perirhinal cortical synapses. At seven and nine months, however, paired-pulse facilitation was evident at intervals from 60 ms to 2000 ms.

It is interesting that those receptor antagonists that altered the wild-type paired-pulse profiles such that they closely resemble the profiles seen in transgenic slices are the group II mGlu receptor antagonist and D_2 dopamine receptor antagonist. Both of these receptors subclasses have been reported as reduced in the R6/2 mouse brain and are therefore likely to be reduced in the R6/1 brain also. Moreover, both of these receptors are negatively coupled to adenyl cyclase and cAMP production, suggesting that this pathway may be particularly susceptible in HD.

The recovery of paired-pulse depression in transgenic slices by a D_2 dopamine receptor agonist is exciting. It suggests that, while the mechanism responsible for normal short-term plasticity is aberrant in transgenic perirhinal cortex, it is not completely absent and can be activated to induce normal function.

4.2.4 Long-term synaptic plasticity

The outcome of the LTD study in transgenic animals was surprising. Given the enhanced expression of LTD at R6/2 hippocampal CA1 synapses (Murphy *et al.*, 2000), one may have predicted that, in a region where LTD is normally expressed i.e.

the perirhinal cortex, LTD may be induced either more frequently or to a greater degree. Conversely, given the intriguing link in the wild-type study that those drugs that have the most profound effect on PPD also block LTD, one may have predicted, in light of the altered transgenic paired-pulse profiles, that LTD would be impaired. At the younger ages studied (approximately two months), the outcome of LFS fitted the first prediction and a large LTD was induced. During development, however, the frequency of LTD induction and the degree there of, decreased until LTD was never seen and low-frequency stimulation induced a small degree of potentiation, thus fitting the later prediction.

Similar to the observation noted with the receptor dependency of paired-pulse depression, antagonists of both group II mGlu and D_2 dopamine receptors blocked LTD induction in the wild-type slices. This further suggests that the common transduction pathway shared by these receptors, the cAMP pathway, is important in the expression of LTD and that interruption of this pathway may be evident in HD.

The changes in synaptic plasticity could also be brought about by the many components of the signalling cascades involved in synaptic plasticity that are reduced in HD. In support of the importance of cAMP regulation in the expression of perirhinal cortical plasticity, mRNA levels are altered from the initial step of this pathway (see introductory table 1.2.2); several subtypes of adenyl cyclase show reduced mRNA levels (Luthi-Carter *et al.*, 2000). A reduction in basal adenyl cyclase means the down regulation in cAMP levels that maybe required for perirhinal cortical LTD would not be achieved and hence failure to express LTD.

The downstream components of the group I mGlu receptor cascade are affected to a greater degree. One of the pathways activated by these receptors is the

DAG pathway, which leads to activation of PKC. PKC mRNA is reduced in both the cortex (Harris *et al.*, 2001) and striatum (Luthi-Carter *et al.*, 2000; Harris *et al.*, 2001) of R6/2 mice. PKC is one of the activators of CREB and hence the expression of genes responsible for the expression of synaptic plasticity. Secondly, group I mGlu receptor activation leads to the production of IP_3. Messenger RNA for the IP_3 receptor is reduced in HD (Luthi-Carter *et al.*, 2000) and hence calcium release from intracellular stores will be affected. Furthermore, the mRNA levels of the downstream targets of calcium released from the endoplasmic reticulum are also reduced. Both CaMKII and the protein phosphatases 1 and 2B (calcineurin) mRNA levels are less in the R6/2 striatum compared to wild types (Luthi-Carter *et al.*, 2000). The balance between the activities of these synergistic molecules maintains appropriate AMPA receptor phosphorylation. Changes in the availability of these molecules could affect the ability to modify the phosphorylation state of AMPA receptors and hence the ability to express synaptic plasticity. Indeed, in the hippocampus of normal rats, a maintained protein phosphatase activity is required for the continued expression of LTD (Mulkey *et al.*, 1993).

The importance of other neurotransmitter receptor classes has not been assessed in the mouse perirhinal cortex. It is known that muscarinic receptors are modulators of LTD expression in the rat perirhinal cortex (Warburton *et al.*, 2003) and the expression of these receptors is reduced in the R6/2 mouse (Cha *et al.*, 1999). Other receptor subclasses are affected in HD, in particular mRNA for CB1 cannabinoid and α2 adrenergic receptors and VSCC are detected at lower levels in the R6/2 striatum (Luthi-Carter *et al.*, 2000). While the roles of these neurotransmitters and channels have not been investigated in the perirhinal cortex,

they are known to modulate plasticity in other brain regions (for example see Harvey & Lacey, 1997; Barbara *et al.*, 2003).

There could also be a presynaptic deficit underlying the synaptic phenotype of the R6/1 perirhinal cortex. As discussed in the introduction, two of the proteins involved in the exocytotic process are altered in models of HD. Firstly, the phosphorylation state of synapsin 1 is enhanced (Liévens *et al.*, 2002) and therefore neurotransmitter vesicles are more likely to be released. Secondly, this situation is compounded by complexin depletion (Morton & Edwardson, 2001; Edwardson *et al.*, 2003), which leads to a reduced inhibition on vesicle release. With an increased probability of neurotransmitter release, it is more likely that depletion of vesicle pools could occur during periods of sustained stimulation such as the conditioning periods used to induce LTD in this study. Given the similarity between the hippocampal electrophysiological phenotypes of the R6/2 and a complexin II knock out mouse (Takahashi *et al.*, 1999; Murphy *et al.*, 2000), it seems likely complexin II deficits may underlie the abnormal synaptic phenotype in the hippocampus and this may also be true for the perirhinal cortex.

In support of a presynaptic deficit are the results obtained in a study of hippocampal long-term synaptic plasticity that indicates an inability to sustain prolonged release during high-frequency trains of stimuli (Usdin *et al.*, 1999). The results of the Usdin *et al.* study were obtained using sharp intracellular recordings in the presence of the activity-dependent NMDA receptor channel blocker MK801 and highlight a reduced decay rate of the NMDA receptor-mediated postsynaptic current in the hippocampus of a knock-in mouse model compared to that in wild-type mice. Given the NR2B specific enhancement of NMDA receptor function conferred by mutant huntingtin (Chen *et al.*, 1999b; Zeron *et al.*, 2001; 2002), while in normal

adult animals the NR2A receptor predominates (Insel *et al.*, 1990; Monyer *et al.*, 1994), an alternative explanation could be applied to the results obtained by Usdin *et al.*. NR2B receptors have approximately half the opening probability of NR2A receptors (Chen *et al.*, 1999a) and this could be reflected as a reduced decay rate using the techniques utilised in the study. In support of this latter explanation of the Usdin *et al.* paper are the results obtained by Murphy *et al.* (2000) which pointed to impairments in NMDA receptor function rather than of transmitter release. Further evidence for an altered NMDA receptor function came from studies of the YAC mouse, which suggested reduced NMDA receptor activity. The results of this study, however, also suggested an impaired ability to sustain transmitter release. It should be noted again that these results were obtained using magnesium-free ACSF, making the data hard to integrate with those obtained in the other models.

It appears, therefore, that the changes in hippocampal synaptic plasticity may be a result of both an altered NMDA receptor function *and* impairments in neurotransmitter release. It is not likely, however, that the results obtained in the perirhinal cortex of the R6/1 mouse are due to an altered NMDA receptor function because LTD in the normal mouse perirhinal cortex is NMDA receptor-independent. Experiments designed to investigate this are, however, yet to be carried out.

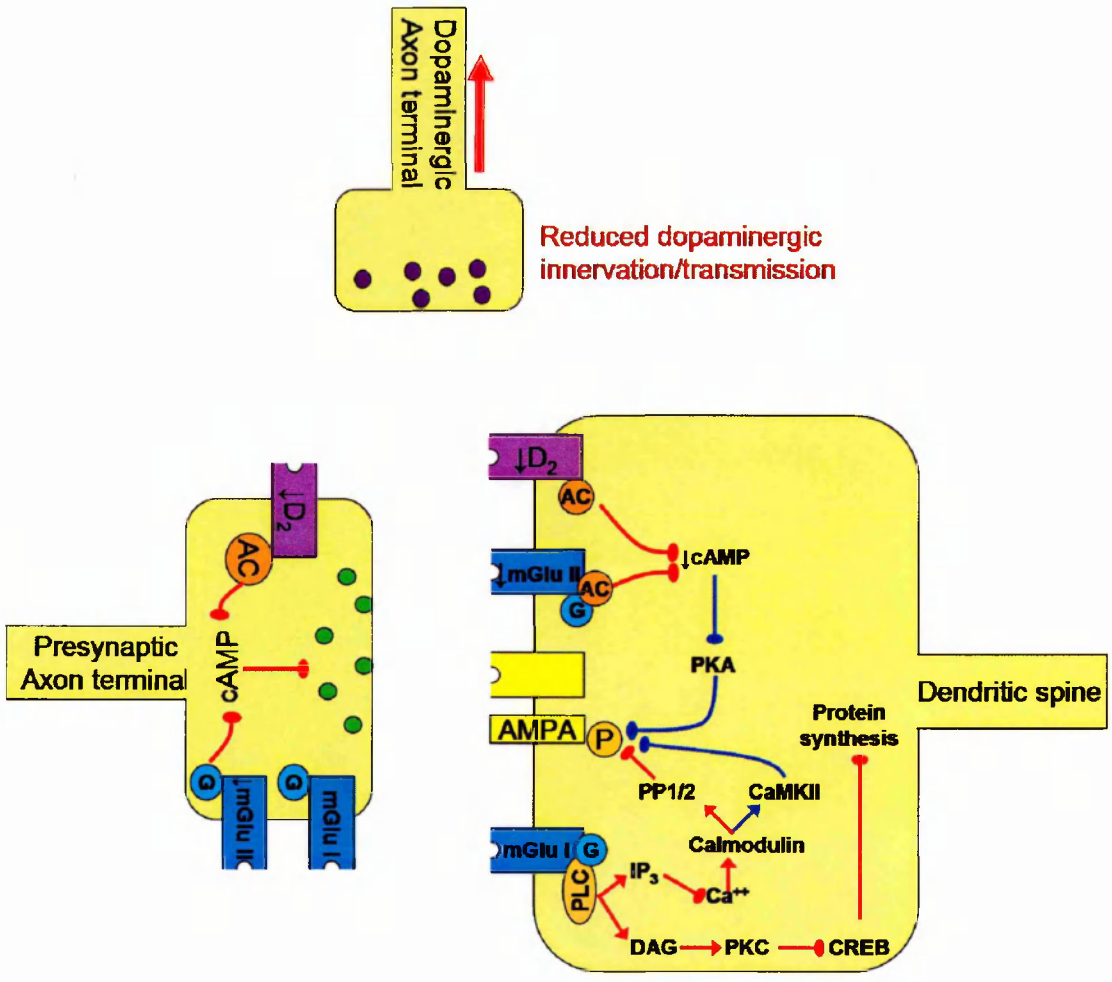


Figure 4.2.1 Proposed mechanisms of altered perirhinal cortical synaptic plasticity in the R6/1 mouse. 1) Reduced dopaminergic transmission could occur through inappropriate activity, dystrophic neurites or other pathological events within the dopamine fibres arising from the VTA. 2) Presynaptic changes could include a reduced expression and/or function of D_2 dopamine and group II mGlu receptors. This leads to less cAMP production, manifest most clearly as a shift from PPD to PPF. 3) Similarly, postsynaptically, the D_2 dopamine and group II mGlu receptors could be reduced, again inappropriately altering cAMP production. There could also be alterations in the downstream cascades of group I mGlu receptors. Lines with a rounded head indicate steps in the normal cascades that are known to be affected in HD. (See figure 4.1.3 for key.)

4.3 Pathological changes in the R6/1 perirhinal cortex

4.3.1 Expression of mutant huntingtin

The layer specific expression of mutant huntingtin within the nucleus is interesting. Previously in the R6 mouse models, mutant huntingtin has been detected either in the cytoplasmic cellular compartment or as aggregates within the nucleus. The detection of a diffuse nuclear stain prior to the formation of inclusions has yet to be reported in the R6 mouse model. In contrast, nuclear staining as been reported in other mouse models of HD, including truncated and full-length transgenic models and knock-in models.

Nuclear staining within perirhinal cortical neurones appeared in an age- and layer-specific manner, becoming evident in the surface cell layers before the deeper layers. Layer-specific nuclear localisation of mutant huntingtin is interesting because this suggests a differential susceptibility of cell types to early pathogenic events within the HD cortex. How a differential propensity for nuclear aggregation is conferred is unclear and is a question that remains unanswered throughout the HD brain.

Due to the coincidental formation of aggregates and the development of the synaptic phenotype in the R6/2 mouse, it had previously been suggested that impairments in synaptic plasticity were underpinned by inclusion formation (Murphy *et al.*, 2000). The data obtained in this thesis suggests otherwise. Altered synaptic plasticity is observed many months prior to the formation of inclusions in the R6/1

perirhinal cortex, a result that was not detectable in the more aggressive R6/2 mouse. It should be noted, however, that the formation of inclusions is likely to occur at ages younger than 7 months. Due to animal availability and time limitations, animals were prepared from 1, 3 and 7 month old animals. Ideally 5 month old animals should also be studied, an age where the motor phenotype and a reduction in perirhinal LTD is detectable. Within the R6/1 striatum, inclusions are detectable as young as 16 weeks (Naver *et al.*, 2003). It is therefore likely that inclusions are present within the cortical mantle at a younger age than highlighted by this study.

4.3.2 Dopamine receptors

All slices incubated with D_2 dopamine receptor antibodies showed a similar pattern of fluorescence. There was a high-intensity band of fluorescence in layer I, while layer II showed only low levels of staining. The pyramidal cell layers III and V showed a second band of relatively high fluorescence, but at a much lower level than the surface layer. Layer VI showed little staining for D_2 dopamine receptors. There was a third intense band highlighted in the white matter. This laminar distribution of D_2 dopamine receptors is in support of the distributions reported by Goldsmith & Joyce (1994) in the rat, but contrary to the earlier report by Richfield and colleagues (1989). The data presented here demonstrates that, during the development of the normal perirhinal cortex, there is an increased D_2 receptor expression in all layers and this is particularly evident in layer I.

This study highlights that the R6/1 perirhinal cortex at 7 months of age has a lower expression of D_2 dopamine receptors than that of age matched wild type littermates. The difference in D_2 dopamine receptor expression appears to be a lack of a developmental increase in expression that is seen in the wild types, but not transgenic animals. In fact, at one month of age, transgenic animals show a trend towards more D_2 dopamine receptors than wild type animals. While the only significance obtained in this section of the study was at 7 months within layer I of the cortex, at all ages there were consistent trends across all cortical layers. That is, at one month of age, all cortical layers showed slightly higher D_2 dopamine receptor expression than did wild types, while at three and seven months, all layers tended to express less receptors.

The pattern of D_1 dopamine receptor expression with the perirhinal cortex is similar to that of D_2. The highest intensity fluorescence was always in layer I, while layer II showed the lowest levels. Layers III, V and VI showed a band of intermediate intensity staining. The white matter showed another band of high intensity fluorescence, but was always less than the surface layer. Again, this is in agreement with the rat study by Richfield *et al.* (1994).

Developmentally, the expression of D_1 dopamine receptors within the perirhinal cortex mirrors that of D_2 receptors. While D_2 receptors increased, there was a down-regulation of D_1 receptors; again, this change was particularly evident in layer I.

There has not previously been a developmental study of the expression of dopamine receptors within the cortex. In wild-type mouse perirhinal cortex there is an age-dependent increase in D_2 dopamine receptors, while D_1 dopamine receptors are

down-regulated and these changes are particularly evident in layer I. The increase in D_2:D_1 dopamine receptor ratio within the perirhinal cortex demonstrates the relative greater importance of D_2 receptors with this brain region. Taken together with the data obtained in the pharmacological experiments presented here and with the evidence that methamphetamine toxicity impairs recognition memory, while sparing spatial memory (Bisagno *et al.*, 2002), it is clear these receptors are important in the normal function of the perirhinal cortex.

4.4 Drug recovery of normal synaptic plasticity in the R6/1 perirhinal cortex

The finding that the D_2 receptor agonist Quinpirole can restore plasticity that resembles that seen in normal mouse perirhinal cortex is interesting and potentially important in the development of future therapeutic strategies. The current dopamine therapies are aimed at controlling chorea by reducing dopamine levels within the striatum. To achieve this, two approaches are taken, that of receptor antagonism and that of monoamine depletion. Both of these approaches lead to a reduction in dopamine transmission throughout the brain. Given that perirhinal cortical plasticity and function is dependent on dopaminergic transmission, preventing dopaminergic activity may be compounding recognition memory deficits that are prevalent in HD (Lange *et al.*, 1995; Hahn-Barma *et al.*, 1998; Lang *et al.*, 2000; Berrios *et al.*, 2002). Therefore, a targeted therapy aimed at increasing dopamine transmission within the cortex may be beneficial in HD. By differentially changing dopamine transmission such that cortical dopamine is increased, while basal ganglia dopamine is reduced, a

treatment may be achieved whereby choreic movements are controlled, but at the same time sparing cortical function.

The fact that LTD and PPD can be restored at both five and seven months of age suggests that, while dopamine receptor expression is reduced, there are still enough receptors to affect normal plasticity. This raises the possibility that the impairment is of dopamine release within the perirhinal cortex rather than a reduced receptor expression and/or function. The primary source of dopamine within the perirhinal cortex is from long projection neurones originating in the mesencephalic ventral tegmental area A10 (Lindvall *et al.*, 1974). The release of dopamine from these terminals may potentially be affected in several ways. Firstly, these projections are particularly long compared to the glutamatergic afferents of the perirhinal cortex that arise in the hippocampus and adjacent cortical regions. Their greater length means there is greater scope for the development of pathogenic events, e.g. the formation of cytoplasmic aggregates and dystrophic neurites. Secondly, mutant huntingtin is known to reduce the expression of neurotrophic factors, including BDNF, that are required for the maintenance and survival of neurones (Zuccato *et al.*, 2001; 2003). If there are reduced neurotrophic factors, the survival of the dopaminergic afferents from the VTA to the perirhinal cortex may be jeopardised. Thirdly, mutant huntingtin is known to directly interfere with axonal transport and vesicle trafficking (for example see Gunawardena *et al.*, 2003; Szebenyi *et al.*, 2003), a process that could be further hindered by the structural changes mentioned above. Therefore, the expression of critical proteins for neurotransmitter release (e.g. voltage sensitive receptors or SNARE proteins) may be reduced at these terminals. Finally, evidence from our lab suggests that the basal properties of VTA dopaminergic neurones may be altered (Dallérac, unpublished data). In wild type

mice, these neurones show pacemaker activity, fire in a regular pattern typical of dopaminergic neurones and show a typical, large afterhyperpolarisation (see also Murphy & Greenfield, 1992). In contrast, in seven month old R6/1 mice, the neurones of the VTA show only a small afterhyperpolarisation and do not fire in a regular pattern. Furthermore, transgenic VTA neurones show impairments in their ability to support continued firing in response to a depolarising current step. Wild type mice fire action potentials throughout a positive current injection, while transgenic neurones fire in a bursting pattern immediately following the onset, but do not maintain firing during the remainder of the current injection. Given this inability to maintain sustained firing by the dopaminergic neurones, it is likely that the perirhinal cortex is receiving inappropriate dopaminergic inputs. With a reduced basal level of dopamine release, the normal function of perirhinal cortical neurones will be altered. The D_2 dopamine subclass of receptor appears the more important both pre- and postsynaptically at glutamatergic synapses of the perirhinal cortex; antagonism of this receptor dramatically alters the paired-pulse profile, while agonism of these receptors hyperpolarises pyramidal neurones and reduces their input resistance. The basal cellular properties of aged transgenic perirhinal cortical neurones fit with a reduced dopaminergic basal modulation *via* D_2 dopamine receptors. The cells are depolarised and show an increased input resistance. Furthermore, synapses show a paired-pulse profile favouring facilitation, again consistent with a reduced level of dopaminergic transmission. The proposed mechanism underlying the electrophysiological phenotype of R6/1 perirhinal cortical neurones and synapses is summarised in figure 4.2.1

Given the co-dependency of perirhinal cortical synaptic depression upon both D_2 dopamine and group II mGlu receptors and also that LTD could be recovered by

the D_2 receptor agonist Quinpirole, recovery of LTD was also attempted using the agonist of group II mGlu receptors (2S,1'R,2'R,3'R)-2(2,3-dicarboxycyclopropyl)glycine (DCG-IV, Ishida *et al.*, 1993). Preliminary experiments were carried out to investigate if activation of group II mGlu receptors could bring about a similar recovery of plasticity. Application of the drug to slices prepared from seven month old transgenic animals did not, however, improve LTD induction (figure 4.4.1). Given the common transduction pathway of this subclass of mGlu receptor and D_2 dopamine receptors, it is surprising that DCG-IV does not improve synaptic plasticity. There are several explanations as to why DCG-IV does not restore LTD at transgenic synapses. Perhaps the most obvious may be that the synaptic phenotype is not a result of an mGlu receptor deficit and therefore activating these receptors does not trigger the appropriate signal cascades required for depression in the perirhinal cortex. It would be interesting to carry out a similar fluorescent histochemical study to the dopamine experiments to look at mGlu receptors in the R6/1 perirhinal cortex. It may very well be the case that these receptors are not lost to a significant degree in transgenic mice. Conversely, it could be that by seven months, the loss of mGlu receptor expression and/or function is too severe and therefore applying an agonist of these receptors is not effective. Again, a histological study may go some way to investigate this.

Yet another possibility is the group II mGlu receptors may normally be located on dopamine terminals where they could regulate the release of dopamine into the perirhinal cortex and hence alter the modulatory role of dopamine within this region. If it is indeed the case that the dopaminergic terminals are non-functional, as suggested by the VTA data mentioned above, activation of group II mGlu receptors may not produce the required response to restore normal plasticity.

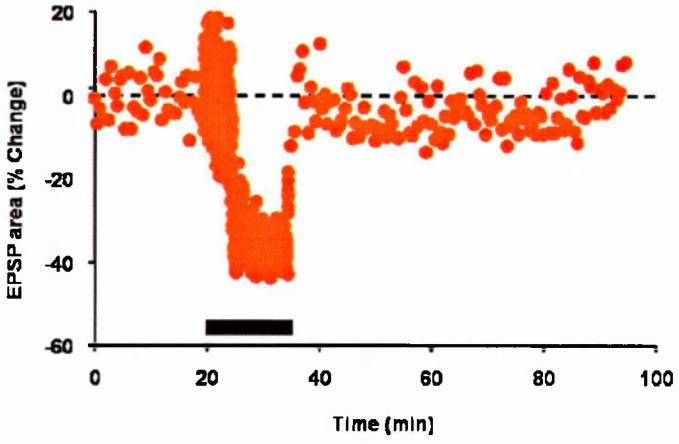


Figure 4.4.1 Pharmacological activation of group II mGlu receptors does not restore LTD at seven month old R6/1 perirhinal cortical synapses. 10 μM DCG-IV, applied to transgenic slices, did not facilitate LTD induction by LFS in the three slices studied. Data points represent the mean EPSP area (% change). Due to the small n value, SEM has not been plotted. n=3(1).

The hypothesis behind these experiments was based on the dependency of normal plasticity in the perirhinal cortex, which suggested that the common mechanism of cAMP down regulation could be affected. It could be the case, however, that the synaptic phenotype is not mediated by a cAMP deficit, but rather is seated at or above the level of the receptor and is purely due to a dopaminergic deficit.

Summarised in figure 4.4.2 are the results obtained in this study compared to those obtained in the R6/2 hippocampus. It is clear that the R6/1 mouse is the superior model for a linear study of the progressive phenotype. The development of many aspects of the phenotype are masked by the aggressiveness of the R6/2 phenotype. For example, the nuclear localisation of mutant huntingtin prior to the formation of distinct inclusions was not seen in the R6/2, while in the R6/1 mouse there was a clear pattern of nuclear translocation. (Data obtained in this laboratory shows a similar pattern of dispersed nuclear staining prior to the formation of distinct aggregates in the R6/1 hippocampus, Milner, unpublished data.) Similarly, the development of the synaptic phenotype in the R6/2 mouse was a single event, with no gradual onset, while in the R6/1 mouse a slower onset was observed, with clearly definable phases. (Again, demonstrated in this lab, the R6/1 CA1 field shows a progressive alteration in LTD that was missed in the R6/2 mouse, Milner, unpublished data.)

It is clear that there is not a common change in synaptic plasticity that occurs throughout the HD brain. While the hippocampus shows a trend towards depression (loss of LTP and aberrant LTD), the perirhinal cortex shows a trend towards potentiation.

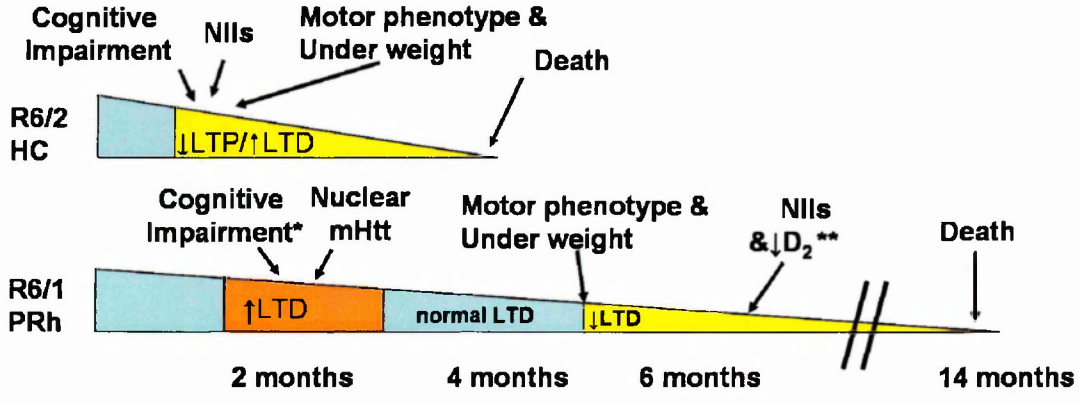


Figure 4.4.2 Summary of phenotypes in the R6/2 and R6/1 mice. The life spans of the R6/2 and R6/1 mice are represented by the blue triangles. Above each age span are indicated the onset of various aspects of the phenotype. * Cognitive impairments as indicated by Mazarakis, 2003; Cybulska-Klosowicz *et al.*, 2004, unpublished data. ** The presence of inclusions and a reduction in D_2 dopamine receptors may be evident at younger ages. The coloured areas within the life spans indicate altered synaptic plasticity within the R6/2 hippocampus (HC) and R6/1 perirhinal cortex (PRh).

4.5 Consequences for the human condition

Long-term depression has been previous proposed to underlie the decremental change in firing rate observed in rats and monkeys during the process of cue familiarisation (for example see Fahy *et al.*, 1993; Zhu *et al.*, 1995; Zhu & Brown, 1995; Xiang & Brown, 1998; Brown & Aggleton, 2001). Further evidence for long-term depression directly underling this change in firing rate are presented here, where LTD of the field response is mediated by a reduction in the probability of the postsynaptic cell firing an action potential. Given this relationship between LTD and recognition memory and that LTD induction is impaired in the perirhinal cortex of the R6/1 mouse, it is likely that the early deficits in recognition memory associated with HD are mediated by a reduction in perirhinal LTD. An alternative mechanism to explain the deficits in recognition memory are provided by the experiments of Thomas Brown *et al.*, which suggest that short-term changes in the firing pattern of perirhinal cortical neurones could underlie memory formation within this region. Therefore, the aberrant expression of paired-pulse facilitation that is highlighted in this study could directly affect the firing patterns of perirhinal neurones and hence mediate the impairment in recognition memory. Indeed, the firing pattern of perirhinal cortical neurones in R6/1 slices is altered. Unlike wild-type neurones, which fire in a regular pattern upon depolarising current injection, R6/1 neurones fire a single action potential and are then quiescent for the remainder of the current pulse. There is an apparent disparity between this result and the observation of PPF in transgenic slices. The expression of PPF suggests an enhanced transmitter release, which could lead to prolonged postsynaptic depolarisation similar to that caused by

current injection and, given the large population-spike component of perirhinal filed potentials, PPF is likely to be expressed as an increase in spiking upon the second pulse. It is surprising that synaptically evoked depolarisation leads to enhanced action potential generation, while cells show impaired ability to support repetitive firing in response to current injection. It is therefore likely that detrimental changes exist both pre- and postsynaptically in the R6/1 perirhinal cortex. Importantly, R6/1 perirhinal neurones do not express long-term decreases in action potential firing in response to a conditioning stimulus. This suggests that deficits in recognition memory seen in the human condition may be mediated by this inability of the perirhinal cortex to support long-term plasticity.

It would appear that dopaminergic deficits contribute to the changes in synaptic plasticity in the R6/1 perirhinal cortex where there is a reduced expression of both D_1 and D_2 dopamine receptors. Similar reductions have been highlighted within the cortex of symptomatic and presymptomatic HD gene carriers (Ginovart *et al.*, 1997; Andrews *et al.*, 1999; Pavese *et al.*, 2003). While these PET studies did not target the perirhinal cortex, it would not be surprising to find altered receptor expression here also. Dopamine has been shown to be important for recognition memory in rats (Besheer *et al.*, 1999; Bisagno *et al.*, 2002; Belcher *et al.*, 2003; Woolley *et al.*, 2003) and, therefore, is likely to also be important in primates including humans. A recently published article in which a reward-based task, shown to be dependent on the entorhinal/perirhinal cortex was investigated. DNA recombination was used to decrease D_2 dopamine binding in monkeys and found to create similar deficits as those caused by bilateral ablation of these cortices (Liu *et al.*, 2004). Given that dopamine receptors are important for cognitive processes

within the perirhinal cortex, it is possible that a reduced dopamine receptor expression in HD patients could mediate early cognitive deficits.

A reduction in dopaminergic transmission may also be mediated by altered activity and innervation by dopaminergic neurones arising in the mesolimbic brain regions. While preliminary data from our lab suggests these neurones to have abnormal basal properties in the R6/1 mouse, the substantia nigra and ventral tegmental area in HD patients have yet to receive an extensive study. The GABA/Substance P containing striatonigral neurones projecting to the substantia nigra are, however, known to be lost at intermediate pathological grades of HD (Reiner *et al.*, 1988; Glass *et al.*, 2000). With an altered GABAergic drive to the dopaminergic neurones it is likely that they have different electrophysiological properties and thus have aberrant activity in HD, which may, in turn, manifest as altered cortical function.

The result demonstrating nuclear translocation of mutant huntingtin correlates with the time course of the electrophysiological phenotype better than the formation of inclusions indicates that aggregates are consequential, rather than causative, of cellular dysfunction in HD. It is possible that inclusions form as a result of excessive mutant huntingtin and these may be neuroprotective at their out start, but may progress to become detrimental to neuronal function and hence lead to cell death. Evidence for a protective mechanism comes from experiments in which cultured rat striatal cells were transfected with mutant huntingtin. As expected, mutant huntingtin led to aggregate formation and cell death. However, prevention of aggregate formation by interfering with the ubiquitination process enhanced the degree neurodegeneration observed (Saudou *et al.*, 1998), suggesting that the formation of aggregates removes the toxic protein from the cell to confer protection.

Indeed, a recently published article demonstrates that the amount of diffuse nuclear staining in cultured rat striatal neurones closely predicts cell death, while inclusion formation correlates with improved cell survival and reduced mutant huntingtin elsewhere in the neurone (Arrasate *et al.*, 2004). Further evidence against a causative role of aggregates are the distributions of inclusions within the human HD brain. While inclusions are prevalent in areas that undergo extensive degeneration (cortex and striatum), they are also detected in areas such as the cerebellum, which are generally spared (DiFiglia *et al.*, 1997).

Summary

Following the identification of the gene and the development of predictive testing, it has become apparent that deficits in recognition memory are discernable many years before the onset of classical symptoms. Aspects of recognition memory are believed to be encoded within the perirhinal cortex by activity dependent decrements in neuronal activity and the cellular mechanism is believed to be that of long-term depression.

In brain slices prepared from the R6/1 mouse model of Huntington's disease, I show that perirhinal cortical long-term depression is aberrantly down-regulated in an age-dependent manner. Interestingly, a synaptic phenotype similar to the that seen in transgenic slices can be recapitulated in wild-type slices using D_2 dopamine receptor antagonists. Moreover, normal cortical synaptic function can be restored in transgenic slices by the application of D_2 dopamine receptor agonists.

In slices prepared from the same animals as those used for electrophysiology, I show using histological techniques that dopamine receptor expression is decreased in the surface layers of perirhinal cortex. Furthermore, this decrease in receptor expression coincides with the nuclear translocation of mutant huntingtin, but not the development of full inclusion bodies. This result differs from the widely held belief that inclusions are directly correlated with pathogenesis and cellular dysfunction. Interestingly, my data is consistent with recent findings suggesting that cell death is positively correlated closely with nuclear mutant huntingtin levels, whilst inclusion bodies correlate more closely with cell survival (Arrasate *et al.*, 2004).

Given that normal function can be restored by the application of dopamine receptor agonists, it would appear that, while there is a reduction in receptor

expression within the cortex, normal dopaminergic function is possible, suggesting a deficit may lie with the dopaminergic input from the mesolimbic system, along side changes within the cortex.

Limitations and future considerations

There are several aspects of the thesis presented here that could be improved or expanded and some of these are discussed below.

The pharmacological experiments presented here could be improved by demonstrating a reversal of any blockade following drug wash-out. This would enable paired t-tests to be carried out, increasing the significance of these results. However, given the relatively large n values and the consistency of these results, there is little doubt in my mind that the results obtained were drug related rather than a result of aplasticity of any given slice.

While the lab was not equipped to carry out patch clamp experiments, many of the experiments presented here would benefit from the study of the underlying synaptic currents. Such experiments may reveal the synaptic currents involved in plasticity within the normal perirhinal cortex and, furthermore, highlight pathological changes underlying the abnormal field response and plasticity seen in transgenic slices.

The results presented here suggest that alterations in the dopaminergic pathway may be an important pathological change within the HD cortex. The data also suggest that therapy targeted at restoring normal dopaminergic modulation within the cortex maybe beneficial to HD patients. Further characterisation of these changes is required in order to assess the feasibility of such a therapeutic approach. Perhaps the key experiments required in this line of investigation is to determine whether the

deficit is at the receptor level or further down the transduction cascade, for example inappropriate adenyl cyclase activity or a reduced basal cAMP level. In order to assess this, the precise role of cAMP in perirhinal cortical synaptic plasticity should be determined. Further investigation of the MAP kinase transduction cascade may yield interesting results, as may the role of metabotropic glutamate receptors in aberrant plasticity in the HD cortex.

The effect of the transgene on LTP in the perirhinal cortex has not been examined. It would be interesting to determine whether LTP is either impaired (as in the hippocampus) or enhanced. While LTD is likely to underlie the decremental change in neuronal firing rate seen in the perirhinal cortex during cue familiarisation, a role for LTP has yet to be suggested. It is possible that the many other cognitive tasks in which the perirhinal cortex is known to be involved (for example fear conditioning) may be mediated by LTP. It would therefore be interesting to not only establish whether LTP is altered in the R6 mouse, but also if fear conditioning is impaired. However, in asymptomatic gene carriers, the recognition of the emotion of fear is normal (Gray *et al.*, 1997), suggesting that aspects of cognitive processes involved in fear are not affected early in the human condition.

A full study of recognition memory in the R6/1 mouse would be beneficial to the results presented here, providing complementary cognitive data to the perirhinal cortical electrophysiological results. These experiments would follow a similar experimental design as a preliminary study carried out in light of the results presented here and performed at the University Department of Physiology, Oxford University by Anton van Dellen and colleagues. The study used two variations of

the open field arena. Version one utilised two objects, while version two used three objects. Mice were allowed to explore the objects for two minutes before being returned to their home cage. When the mice were returned again to the open field arena, one object had been replaced by a novel object. The time spent exploring the novel or familiar object(s) was then assessed. While the results obtained from these experiments showed a trend towards presymptomatic mice being impaired on these tasks, they failed to reach significance. The failure to reach significance is likely to be seated in the small number of animals in the transgenic group; a result of the experimenters being blind of genotype.

Questions still surround the spike-LTD results, particularly within the R6/1 perirhinal cortex. The relationship between the depolarised membrane state of transgenic cells and the lack of LTD requires further investigation. This could be addressed by hyperpolarising transgenic cells to −80 mV, which may restore the ability to induce LTD. Alternatively, as shown in this thesis, depolarisation of normal perirhinal neurones to −67 mV does not block the induction of spike LTD. This result suggests factors other than depolarisation to be the cause of aplasticity in the R6/1 perirhinal cortex.

Importantly, the results presented here should be confirmed in other mouse models of HD. While the R6/1 mouse provides a superior model over the R6/2 mouse for studying the progressive phenotype seen in HD, other transgenic mice may offer more faithful models. In particular, the R6 stain of mice contain only a truncated gene and, therefore, the effects of the full-length mutant gene maybe missed. Therefore, the novel findings presented in this thesis should also be investigated in

other mouse models. Two models that may provide good complementary data are the YAC72 mouse and the knock-in mice carrying 71 and 94 CAG repeats. The YAC mouse provides a model carrying the full-length gene, thereby also modelling earlier events in disease pathogenesis, such as cleavage. The second model, the knock-in mouse, has previous reports of aberrant neuronal properties and plasticity in the corticostriatal pathway similar to those seen in the R6/2 mouse (in particular see Levine *et al.*, 1999) and may, therefore, show similar changes in the perirhinal cortex.

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Acknowledgements

I thank my supervisor, Dr. Kerry Murphy, for his enthusiasm, encouragement and inspirational discussion throughout my Ph.D. I would also like to thank my co-supervisor, Prof. Mike Stewart, for his support.

Secondly, I wish to thank the other lab members, Austen Milner, Jon Spencer Glenn Dallérac and Sarat Vatsavayai, for helping make my time in the lab both productive and highly enjoyable.

I also thank everyone in the Department of Biological Sciences, in particular Jacki Brown, Verina Waights, Dawn Sadler, Karen Evans and Steve Walters for their friendship, advise and excellent technical support.

Finally, I thank my family and friends, particularly my partner James and my mum and dad, for their love and affection in everything I do.

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